Development of animal model, vaccines, and diagnostics for Schmallenberg virus

by

Abaineh Dagne Endalew

M.S., University of Minnesota, 2010

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

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Abstract

Schmallenberg virus (SBV) is a novel orthobunyavirus in the Simbu serogroup, genus *Orthobunyavirus,* family *Peribunyaviridae,* and order *Bunyavirales*. The virus emerged in late 2011 near the German/Dutch border region and was mostly associated with a mild transient disease of sheep and cattle. It is transmitted by biting midges (*Culicoides* species) and causes abortions, stillbirths, and congenital defects in naïve pregnant sheep and cattle. SBV has spread throughout most of Europe. However, to date, the initial introduction route of the virus to Europe is poorly understood. Consequently, SBV poses a threat to other countries like the US, where competent insect vector species and susceptible livestock populations exist and extensive trade with Europe is conducted. To this end, research work was conducted with the following three aims: (1) development of a large animal model for SBV infection; (2) subunit vaccine development for SBV; and (3) development and evaluation of Schmallenberg disease diagnostics. This dissertation contains four chapters. The first chapter provides a literature review on SBV emergence, taxonomy, replication, transmission, pathogenesis, diagnosis, and control. The remaining three chapters contain the research conducted to address the above-mentioned three study aims.

In chapter two, the clinical, virological, and serological responses in two ruminant models, i.e., cattle and sheep, are presented. Infectious serum was shown to be the best inoculum in both species when compared to infectious cell culture supernatant and brain homogenate. The virological and serological responses to SBV were more apparent in cattle than in sheep. Thus, cattle are recommended as the better SBV infection model for the evaluation of vaccines and diagnostics. Chapter three presents the immunogenicity and efficacy of a baculovirus-expressed subunit vaccine composed of SBV glycoproteins C and N. After vaccination, SBV Gc-specific antibody response was detected in all vaccinated animals. Neither of the vaccines conferred

protection against SBV challenge infection. Therefore, future studies should focus on better understanding of the difference in post-translational modifications on Gc protein in different expression systems and subsequent conformational and stability conditions that are crucial for its immunogenicity.

Using the different reagents (sera, recombinant proteins, and tissues) generated in the SBV animal model development and vaccine experiments, we tried to develop and evaluate serological and molecular diagnostic assays for SBV. Among the different SBV proteins evaluated for SBV antibody detection, N and Gc were the most reactive antigens, followed by Gn. In the indirect ELISA experiments, the SBV-infected cell lysate was the least reactive. Among the three commercial nucleic acid extraction kits, the GeneReach total RNA extraction kit yielded approximately 10× more SBV *in vitro* transcribed RNA than the Qiagen and Applied Biosystems extraction kits. Multiplex RT-qPCR using in vitro transcribed SBV RNA resulted in favorable amplification of the L and M genes than the S gene.

In order to facilitate full genome sequencing of SBV isolates for diagnostic purposes, we established a full genome sequencing approach for three different SBV isolates: FLI-serum, KSU-serum, and KSU-serum cell passaged, using Next Generation Sequencing. Overall, SBV genomes between each of the tested samples have over 99.9% homology, indicating a low level of molecular evolution at this level of investigation.

Overall, these studies have highlighted (i) that cattle is the preferred animal model to evaluate SBV vaccines and diagnostics, (ii) the need for identifying the right protein expression system that ensures proper conformation and stability and, finally, (iii) that both M and L segment PCRs are more specific than the S segment PCR, which is relevant especially in areas where other Simbu serogroup viruses are endemic.

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Approved by:

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Dedication

To my wife Yete and my daughter Abigal who have given me tremendous support and encouragement during my PhD study. This wouldn't have been possible without their unflinching help and love.

Chapter 1 - Literature review

1.1 Introduction

Viruses belonging to the Bunyavirales order are assigned to five families: *Hantaviridae*, *Peribunyaviridae*, *Nairoviridae*, *Phenuiviridae*, and *Tospoviridae*. These viruses were formerly listed under the *Bunyaviridae* family and encompass RNA viruses that have medical, veterinary and agricultural importance (Adams et al., 2017). With over 350 identified viruses distributed worldwide, these viruses represent a global threat to livestock, agricultural systems and human public health. Many cause serious diseases with high mortality rates in humans and domestic animals, such as fatal hepatitis, encephalitis, and hemorrhagic fever (Junglen & Drosten, 2013). Bunyavirales are unique in the way that they infect a large range of hosts, including vertebrates, invertebrates and plants. The increasing frequency of bunyavirus outbreaks over the last decade make these virus members as the emerging pathogen group. No vaccines or treatments are currently approved for human use. Some bunyaviruses are classified as potential biological weapons and listed as high-priority pathogens by the World Health Organization (Albornoz et al., 2016).

With the exception of the Hantaviruses, members of the order Bunyavirales are transmitted primarily via arthropod vectors. Recently, concerns have been raised over the ongoing geographic spread of several of these vectors, notably the hyalomma tick that is associated with Crimean–Congo hemorrhagic fever virus (CCHFV) transmission (Hauman and Feldmann, 2018), and the Aedes and Culex mosquitoes, which are important vectors for the hemorrhagic fever-causing Rift Valley fever virus (RVFV) (Tran et al., 2013). Some of these vectors are migrating and establishing a presence in more northerly regions of Europe, possibly due to changes in the global climate (Maltezou & Papa, 2010). It is widely accepted that once the vector is established in a new

environment, the viruses to which it is susceptible will ultimately follow. Therefore, exposure of naive human and animal populations to some of these virulent pathogens seems inevitable (Walter & Barr, 2011).

Bunyavirales are characterized by the possession of a tripartite negative-sense ssRNA genome that encodes four structural proteins. The large (L) segment encodes the viral RNA-dependent RNA polymerase or L protein, the medium (M) segment encodes the virion glycoproteins Gn and Gc, and the small (S) segment encodes the nucleoprotein (N). Non-structural proteins NSm and NSs are encoded on the M and S segments, respectively (Elliott et al., 2013).

1.2 Bunyavirales and reassortment

When two closely related bunyaviruses infect the same susceptible cell at the same time, their genome segments can be mixed and incorporated into the progeny viruses (He and Ding, 2012). These mixed viruses are called reassortant viruses. Segments may combine to generate chimeric segments formed from homologous portions of homotypic or possibly heterotypic bunyaviruses as observed in the newly emerged severe fever with thrombocytopenia syndrome virus from China and other viruses such as Crimean-Congo-hemorrhagic fever virus and Hantaviruses (Briese et al., 2013).

1.3 Orthobunyavirus

The genus *Orthobunyavirus* contains at least 170 named viruses that have been conveniently divided into 18 serogroups on the basis of cross-reactivity in complement fixation, neutralization and hemagglutination-inhibition assays. The Simbu serogroup contains 25 viruses, of which Akabane, Aino, Sathuperi, Peaton, Shamonda and Shuni viruses are known to be

teratogenic in ruminants in Africa, Asia and Australia. SBV represents the first Simbu serogroup virus that has been reported in Europe. Simbu serogroup viruses are transmitted by midges (Akabane, Aino, SBV, Shuni, Shamonda in animals) and by both mosquitoes and midges (Oropouche in human). Analysis of midges trapped in Denmark, Belgium, Poland, and the Netherlands) has shown evidence of SBV infection (De Regge et al., 2012; Elliott et al., 2013; Rasmussen et al., 2012; Larsaka et al., 2013; Elbers et al., 2013, 2015) showed the competency of these vectors for SBV replication and transmission.

1.4 Arthropod transmission

Most orthobunyaviruses are transmitted by mosquitoes or Culicoides midges, although a few are vectored by ticks and the Kaeng Khoi virus is transmitted by bed bugs. In general, only one or a very few arthropod species are competent to transmit a particular orthobunyavirus, and strict vector preference is observed even in regions where different viruses and vectors coexist (Beaty & Calisher, 1991). Female mosquitoes acquire the virus when feeding on an infected host, and infected mosquitoes show few or no adverse signs of infection (Patrican & DeFoliart, 1985). Mosquitoes become persistently and systemically infected, and in some cases, vertical transmission to offspring has been reported (Beaty & Calisher, 1991). Transovarial transmission is an important mechanism for the maintenance of some viruses (such as La crosse virus) during the winter months and can result in infected male mosquitoes that transfer the virus to females during mating (Thompson & Beaty, 1977). Culicoides midges are among the smallest blood-sucking flies, with body lengths that rarely exceed three millimeters (Mellor, et al., 2000). Bluetongue, African horse sickness, and Akabane diseases are amongst the common animal diseases recognized to be caused by viruses and to be transmitted by Culicoides species (Verwoerd

2012). To date, the only arbovirus identified as being primarily transmitted by Culicoides to and between humans is Oropouche virus. This member of the genus *Orthobunyavirus* causes major epidemics of febrile illness in human populations of South and Central America and the Caribbean (Carpenter, et al. 2013). Various studies in France (Balenghien, et al., 2014); the Netherlands (Elbers, et al., 2013); Belgium (De Regge et al., 2012); and Italy (Goeffredo et al., 2013) confirmed the role of *C. obsoletus* as a highly probable vector of SBV in northern Europe. This species is among the most abundant livestock-associated species in the region (Meiswinkel et al., 2008; Carpenter et al., 2009). Although Akabane and Aino viruses were initially isolated from mosquitoes, these seem not important for their transmission (Charles, 1994). SBV was not detected in mosquitoes in different studies and *Culex* pipiens mosquitoes showed no vector competence for SBV under laboratory conditions (Wernike et al., 2011; Scholte, et al., 2014; Manley, et al., 2015).

1.5 Pathogenesis in vertebrate host

Infection is initiated by the bite of an infected arthropod and the virus spreads to striated muscle, where extensive replication occurs before entering the circulation and causing viremia (Taylor & Peterson, 2014) . Some of the viruses in the genus such as Akabane, Aino, and SBV are capable of causing encephalitis in fetuses with the typical arthogryposis-hydoroencephaly syndrome (AHS) following transplacental infection (Kurogi et al., 1978; Tsuda, et al. 2004; Veldhuis et al., 2013; Wernike et al., 2013, 2014; Konig, et al., 2019). Infection of the central nervous system (CNS) is age-related and juvenile animals seem to be more susceptible than adults (Taylor & Peterson, 2014).

1.6 Molecular virology of orthobunyaviruses

Orthobunyavirus have a genome consisting of three segments of single-stranded RNA with negative polarity, termed large (L), medium (M), and small (S) segments. The virions are relatively simple in their overall composition and comprise just four structural proteins: two surface glycoproteins, termed Gn and Gc, and two internal proteins, the N (nucleocapsid) protein and the L protein, the viral RNA-dependent RNA polymerase (RdRp). The three genomic RNA segments are encapsidated by the N protein to form ribonucleoprotein (RNP) complexes that associate with the RdRp and are contained within the lipid envelope of the particle (Fig. 1-1). Electron microscopy analyses of purified virions showed spherical to pleomorphic particles that were ~90 nm in diameter, with a double membrane envelope and a fringe of spikes that were predicted to be the glycoproteins (Obijeski et al., 1976; Hellert et al., 2019).

The average size of each genome segment is generally consistent within the orthobunyavirusgenus, and about 6.9 kb for the L segment, 4.5 kb for the M segment and 1.0 kb for the S segment. The terminal nucleotides at the 3′ and 5′ ends of each segment are complementary, which permits the formation of a ‘panhandle’ structure that functions as the promoter for both the transcription and replication of each segment (Barr et al., 2003).

Although the lengths of each coding region, and hence the sizes of encoded proteins, are relatively conserved among different viruses within this genus, there is much more diversity in the length and sequence of the untranslated regions (UTRs) (Elliott, 2000). In addition to functioning as promoters, the UTRs are also required for the encapsidation of the genomic RNA by the N protein, the termination of mRNA transcription and packaging of the RNP into virus particles (Osborne & Elliott, 2000).

The genomic RNA segments are single-stranded and have a negative-sense polarity, which means that the genomic RNA must be transcribed into positive-sense mRNAs for translation; this occurs immediately after infection because the virus carries its RNA polymerase complex in its virion (Fig. 1.1). The S segment encodes the N protein, the M segment encodes the two external glycoproteins Gn and Gc, and the L segment encodes the RdRp (Elliott, 2014). The glycoproteins are encoded as a precursor polyprotein that also contains the non-structural protein NSm. The coding order of the M segment mRNA is Gn–NSm–Gc (Fazakerley et al., 1988), and it is co-translationally processed by host proteases to produce the three proteins. The S segment of most orthobunyaviruses beside the N protein encodes a non-structural protein, NSs, which is within the N protein-coding sequence and is translated from the same mRNA using an alternative AUG initiation codon (Fuller et al., 1983) in a process known as leaky ribosomal scanning. Both the NSm and NSs proteins are mostly dispensable for virus replication. While NSm seems to be involved in virus assembly and budding, NSs plays an important role as a virulence factor countering host innate immune responses (Weber et al., 2002; Blakqori, et al., 2007; Kraatz, et al., 2015).

1.7 Replication cycle

Cell entry involves interactions between the surface glycoproteins Gn and/or Gc and cell surface receptor; the receptor (or receptors) are unknown, although it has been reported that Germiston Orthobunyavirus entry is promoted by DC-SIGN (Lozach et al., 2011). Although DC-SIGN is highly expressed on dermal dendritic cells and is a good candidate for entry following transmission by an arthropod bite, co-receptors are probably involved in this process, as these viruses infect a wide range of tissues (Elliott, 2014).

Several studies have suggested that Gc is the primary protein involved in virus attachment to both vertebrate and invertebrate cells (Hacker & Hardy, 1997), but it has also been suggested that the smaller Gn protein of LACV functions as the attachment protein for mosquito cells (Ludwig et al., 1989). Mammalian cells are entered through the early endosome in a Rab5-, dynamin-and clathrin-dependent manner, requiring a drop in pH and serine protease activity (Hofmann et al., 2013; Hollidge et al., 2012).

After the release of the RNPs into the cytoplasm, the first event is primary transcription, which involves primer-dependent synthesis of viral mRNAs and results in mRNAs that contain 12–18 host-derived nucleotides at their 5′ ends (Patterson et al., 1984). Orthobunyavirus mRNAs do not have detectable poly (A) tracts at their 3′ ends. The 3′ ends of the viral mRNAs have the potential to form stem–loop structures that may be involved in promoting efficient translation in a poly (A)-binding protein-independent manner (Blakqori et al., 2009).

Orthobunyavirus transcription requires on-going translation in the host cell, which is a unique feature among negative-sense RNA viruses (Kolakofsky et al., 1987). The template for genome replication is full-length positive-sense RNA (known as the antigenome), which is produced in a primer-independent manner from the negative-sense RNA genome. The molecular basis for the switch from transcription to replication is unclear, but the level of N protein in the cell is important, as sufficient N protein must be available to encapsidate nascent antigenomes and genomes during replication. Only the viral RdRp and N proteins are required for transcription and replication (Dunn, Pritlove, Jin, & Elliott, 1995). Whether different isoforms of the polymerase carry out these different activities has not been determined yet. In cells that are co-infected with two different orthobunyaviruses, progeny virions may contain genome segments that are derived

from both parents; this process is known as genome reassortment and can lead to reassortant viruses that have altered biological properties (Elliott, 2014).

1.8 Orthobunyavirus immunology

Being negative-strand RNA viruses, orthobunyaviruses do not produce long stretches of double-stranded RNA (dsRNA), a prominent, immune-active feature or pathogen associated molecular pattern (PAMP) of other types of viruses (Weber et al., 2006). The panhandle structure, however, is a virus-specific trait recognized by a host sensor, the cytoplasmic pathogen recognition receptor (PRR) RIG-I (Habjan et al., 2008; Weber et al., 2013). RIG-I is a DExD/H type helicase that binds short dsRNA structures containing a 5'ppp end (Yoneyama et al., 2015).

The panhandle forms an imperfect dsRNA and is hence not an ideal RIG-I ligand (Schlee, 2013; Weber & Weber, 2014). Nonetheless, RIG-I is able to bind the panhandle structure via nucleocapsids just after they enter the cytoplasm, forming a diamond-ring-like structure with the nucleocapsids as "ring" and RIG-I oligomers as "diamond" (Weber et al., 2013; Weber et al., 2015). The ensuing signal transduction eventually activates the transcription factor IRF-3 (among others), resulting in production of antiviral and pro-inflammatory cytokines, mostly type I interferons (IFNs). Type I IFNs, in turn, activate more than 300 so-called IFN-stimulated genes (ISGs). Some products of ISGs have direct antiviral activity, e.g., Mx, IFIT, ISG20, viperin, the 2-5 OAS/RNaseL system, or PKR (Mukherjee et al., 2013; Taylor & Peterson, 2014).

The NSs of orthobunyavirus is entirely embedded in the gene of the nucleocapsid protein and is identified as a virulence factor and suppressor of IFN induction. A genetically modified Bunyamwera virus and SBV (lacking both NSs and NSm) grew less efficient in both interferon competent and defective cells and type I interferon receptor knock-out mice than the wild-type

virus. However, mice infected with the NSm deleted SBV had more survival rate than mice infected with the wild type virus (Weber et al., 2002; Kraatz, et al., 2015).

1.9 Simbu serogroup viruses

This serogroup include viruses such as: Akabane, Aino, Peaton, Sathuperi, Shamonda, Douglas, and the newly emerged SBV; all are arthropod-borne (arbovirus) that are transmitted principally by *Culicoides* midges. A number of Simbu serogroup viruses have been found to be present in different parts of the world, including Africa, Asia, Australia, Israel, and lately in Europe (Della-Porta et al., 1977; Parsonson et al., 1977; St George, et al., 1979; Shimshony 1980; Golender, et al. 2015; Hoffmann et al., 2012). They have been isolated from domestic and wild animals as well as from respective vectors. Akabane virus has been the most recognized virus in this group together with Shamonda and Aino virus. These viruses are characterized by causing abortion, still birth and congenital abnormalities in ruminants (Tsuda et al., 2004; Kirkland et al., 2015; Bilk et al., 2012; Hoffmann et al., 2012).

1.10 Schmallenberg virus

1.10.1 Discovery and genome structure

In the autumn 2011, a new disease characterized by a drop in milk production, hyperthermia, and diarrhea was reported in dairy cows in north-western Germany and the Netherlands and no known pathogen was identified in samples from symptomatic cattle (Hoffmann et al., 2012). In November 2011, the Friedrich-Loeffler Institut (FLI) in Germany detected viral RNA belonging to a new virus in a pool of blood samples from clinically affected

dairy cows using a metagenomics approach. This new virus was called Schmallenberg virus (SBV) after the place where the blood samples from cows were collected (Hoffmann et al., 2012).

SBV sequence analysis revealed similarities with other viruses such as: Akabane, Aino, and Shamonda, all belonging to the genus *Orthobunyavirus* from the *Peribunyaviridae* family. SB virions, like typical species of the *Peribunyaviridae*, are spheric enveloped particles with short surfaceprojections and thin filamentous nucleocapsid strandsand approximately 80–120 nm in diameter (Fig. 1.1a). The negative-stranded tripartite RNA-genome of orthobun-yaviruses comprises of S, M, and L segments (Fig. 1.1b) (Walter and Barr, 2011; Chowdhary et al., 2012). The open reading frames (ORF) of the L, M and S segment comprise 6765 nt, 4212 nt and 702 nt, respectively. The size of the deduced polymerase L protein is 2254 amino acids (aa) with a molecular weight of about 261 kDa. The deduced length of the M-derived polyprotein (Gn + NSm + Gc) is 1403 aa with a molecular weight of about 159 kDa (Doceul et al., 2013). Gn is supposed to comprise aa 1 to 300. The aa 301 to 480 are ascribed to NSm, and Gc is represented by aa 481 to 1404 (Fischer et al., 2013). The nucleoprotein is 233 aa long with a molecular weight of about 26 kDa (Doceul et al., 2013). The S and the M segment of contain information for two nonstructural proteins, called NSs and NSm, respectively. NSs (~12kDa) is encoded in the form of an overlapping reading frame within the nucleoprotein gene while the NSm (~16k Da) protein is located between those of the Gn and Gc proteins and produced by cleavage of the polyprotein (Walter & Barr, 2011). NSm seems to be involved in viral replication for some bunyaviruses (Shi et al., 2006). However, it must be assumed that this protein also exerts a number of other functions which have not been elucidated yet. Recent report indicated that SBV-NSm is dispensable for the generation of infectious virus; however, deletion of part of the non-hydrophobic domain IV caused an altered distribution pattern of this protein and lacked co-localization with Gc (Kraatz et al.,

2018). NSs on the other hand has been shown to antagonize type I interferon synthesis in mammalian host cells thereby promoting viral replication (Eifan et al., 2013). . In cattle, the NSm deletion mutant caused comparable viremia and seroconversion to that of the wild-type virus, unlike the NSs and the combined NSs/NSm deletion mutant which caused no detectable virus replication (Kraatz et al., 2015).

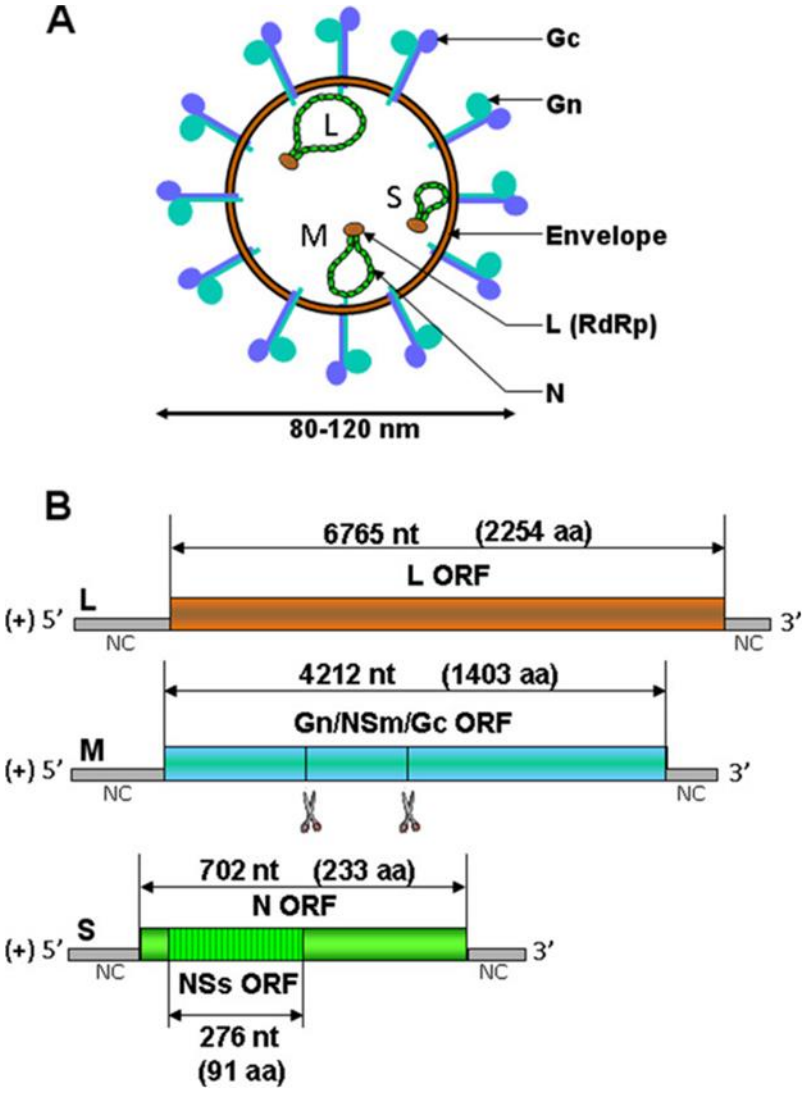

Figure 1.1 Schematic representation of a generic bunyavirus particle (A) and SBV antigenomes (B). (Adapted from Doceul et al., 2013).

1.10.2 Immunity

Schmallenberg virus seems to spread quickly and induces a solid protective immunity (Wernike et al., 2015). It was shown that within a two months period several cattle herds within a large area became infected and up to 90% of the cattle displayed measurable protective anti-SBV antibodies for at least 6 years after infection (Wernike et al., 2018). Calves born from SBV infected cows are mostly protected from infection for six months and a direct correlation between the antibody titer of the dam and the titer of the corresponding calf was observed (Elbers et al., 2014). SBV-infected cattle showed a measurable increase of IgM+ B lymphocytes until day 10 post infection, and anti-SBV antibodies were detected 14 days post infection. CD8+ T lymphocytes were not detected after the first infection indicating virus clearance seems to be independent of T-cell mediated cytotoxicity. However, these cells appear to be involved in the antiviral defense when previously infected animals are exposed to the virus for a second time. The number of CD4+ T helper cells was found to be decreased between day 3 and 7 post infection. Interestingly, the SBV genome was not detected in white blood cells (Wernike et al., 2012).

Differential gene expression analysis in bovine fibroblast infected with wild-type SBV and a NSs-deleted mutant indicated that only nine genes are differentially expressed. On the other hand, 649 genes were differentially expressed in SBV delNSs infected cells compared to un-infected cells and many of these were known antiviral and IFN-stimulated genes. The major molecules involved in viral RNA recognition include DDX58 (RIG-I), TRIM25, IFIH1, MDA50, PKR, and TLR3 which were highly up-regulated in SBV delNSs infected cells (Blomstrom et al., 2015).

1.10.3 Epidemiology

First reports of an unidentified disease of dairy cattle were obtained from farms located in the Netherlands and Germany in the summer of 2011 (Hoffmann et al., 2012). The major clinical signs in affected cattle were diarrhea, drop in milk production, and hyperthermia. In November 2011, the Friedrich Loeffler Institut (FLI) demonstrated that a novel pathogen was responsible for this disease (Hoffmann et al., 2012). The virus was named Schmallenberg virus in reference to the German city of Schmallenberg where the samples were collected. Shortly after, large number of congenital malformations, featuring an arthrogryposis and hydranencephaly (AG-HE) syndrome, in newborn lambs, kids, and calves associated with SBV infections were reported in Central Europe (Luttikholt et al., 2014). Both, Belgium and the Netherlands reported cases of stillborn lambs and newborn lambs rapidly dying with arthrogryposis and brain abnormalities. The affected farms were mainly located in the north of Belgium close to the Dutch border (De Regge et al., 2013). The disease outbreak spread rapidly to England within three months of initial outbreak with the southernmost counties of England all reporting the disease in 2012 (EFSA, 2012). In March of 2012, Spain reported the first case of SBV infection when one ovine fetus was born with congenital lesions indicative of SBV infection (Rodriguez-Prieto et al., 2016). The high SBV seroprevalence (up to 98.03%) in sheep and cattle in geographic areas having reported SBV cases in 2011 and early 2012 was expected to limit reemergence of the virus in 2012 (Elbers et al., 2014). However, a novel SBV episode occurred during summer 2012 in previously affected regions. In 2012, SBV reemerged in Belgian sheep as it was demonstrated serologically by serum neutralization assay and virus detection using RT-PCR (Claine et al., 2013). In 2012, new cases were observed in cattle, sheep, and goats in Germany in areas that were not affected in 2011, proving the ability of the virus to survive and recirculate after the winter period (Conraths et al., 2013). In 2012 and 2013,

SBV spread across Eastern Europe. In late September 2013, a total of 27 European countries were considered endemic for SBV (Claine et al., 2015).

A third SBV episode was reported in late 2014 in the Netherlands and Germany when SBV was detected in malformed calves and lambs (Wernike et al., 2015). In April 2016, SBV RNA was detected in three aborted calves from Belgium, indicating SBV circulation in 2015 (Delooz et al., 2016). After several years of low, or no circulation of SBV in the UK and France, sheep and cattle once again became infected 2017 and 2018 (Gache et al., 2016; Stokes et al., 2018).

1.10.4 Transmission and host range

SBV spreads rapidly, estimated to be 1 km per day and 6 days from region to region (Sedda and Rogers, 2013). The virus has been reported in climate ranges from the Mediterranean basin (Italy and Spain) to more than latitude 60° north (Norway) (Balseiro et al., 2015). Spread of the virus has tended to be from an infected region to a nearby region, rarely exceeding 200 km at a time (Afonso et al., 2014). Mathematical modelling has replicated the spread of SBV across Europe, uncovering a high vector competence and high replication rate for temperatures common in Europe (16–34° C) (Gubbins et al., 2014). The origin of SBV is still unclear, but at least two studies have demonstrated SBV cross-reactive antibodies (along with antibodies against several other Simbu group viruses) in cattle in Africa prior to the European outbreak (Blomstrom et al., 2014; Molini et al., 2018). Antibodies against a Simbu serogroup virus related to Aino virus were identified in Jordan in 2013 in cattle, sheep and goats on farms where clinical signs similar to those caused by SBV had been observed (Abutarbush et al., 2015). Several historical and recent reports have highlighted that viruses from the Simbu group, many of which have teratogenic potential, circulate within the Mediterranean basin (Lievaart-Peterson et al., 2012; Azkur et al., 2013; Chaintoutis et al., 2014; Yilmaz et al., 2014; Golender et al., 2015, 2018). At the height of the

original outbreak in Europe, herds and flocks in Northern Europe were reporting essentially all animals seroconverting to the virus at herd level (98.5-99.8% in adult cattle and 89% in sheep) (Méroc et al., 2013; Veldhuis et al., 2013).

Most of the bunyaviruses present in Asia and Africa are transmitted by an insect vector (biting midges or mosquitoes) (Rasmussen et al., 2012). When SBV emerged in 2011 in Europe, biting midges of the Obsoletus species complex of the ceratopogonid genus *Culicoides* were rapidly identified as SBV vectors. Indeed, viral genome was detected in different *Culicoides* spp. (*Culicoides dewulfi*, *Culicoides chiopterus*, *Culicoides punctatus*, etc) caught as early as summer and autumn 2011 in Belgium, (De Regge et al., 2012), Italy, (Goffredo et al., 2013), the Netherlands, (Elbers et al., 2013) and Denmark (Rasmussen et al., 2012). Under laboratory conditions, SBV replication and dissemination were detected in *Culicoides sonorensis* (De Regge et al., 2012; Veronesi et al., 2013*). Culicoides* biting midges are implicated in transmission of several viruses of the Simbu serogroup. They were recognized as major vectors of bluetongue virus serotype 8 (genus *Orbivirus* from the family of *Reoviridae*) in northern and central Europe during the 2006 outbreak of bluetongue disease (Lehmann et al., 2012). However, the vector competence for SBV may exceed rates recorded for BTV either in the number of species capable of transmitting the virus or in the proportion of individuals within a species (European Food Safety Authority, 2014).

In adult animals, transmission of SBV is through insect vectors, whereas fetuses can be infected transplacentally leading to observations of congenital deformities if viral infection occurs during the critical period of gestation (time period when abortion and malformation of fetuses could happen). However, there is no evidence of horizontal transmission. Besides vector-borne transmission being the most common route of SBV infection, researchers have demonstrated that

SBV RNA-positive bovine semen could contain infectious SBV (Rasmussen et al., 2012; Schulz et al., 2014). Infections via mucous memberane has not been reported so far, hence venereal transmission of the virus from infected semen is unlikely unless intrauterine lesions exist thereby virus enters the circulation. No evidence of infection of the developing embryo has been reported so far (Schulz et al., 2014).

Numerous species have been recognized as susceptible species to SBV infection. Table 1.1 summarizes the animal species in which direct and/or indirect SBV detection was performed and clinical expression in adults and/or their offspring was observed. It appears that clinical features of SBV infection have only been observed in domestic ruminants (cattle, sheep, and goats). However, there was a single report of SBV genome detection in the serum of an elk calf in 2012 from Poland (Larska et al., 2013) and in Mouflon (Wernike and Beer, 2017). The indirect detection of the pathogen (serological reaction to SBV) has only been performed in wild ruminants (eg, alpaca, buffalo, bison), zoo (eg, kudu, zebra, oryx), and some other mammalian species (eg, horse, wild boar), while in dogs, virological and serological evidences of SBV infection have been detected. Experimental SBV infection in embryonated chicken eggs resulted in mortality and stunted growth and musculoskeletal malformations (Collins et al., 2018). Due to their suggestive role in the epidemiology of Akabane virus, the role of pigs in the epidemiology of SBV was evaluated. Experimentally SBV-infected piglets showed only seroconversion underlining the inability of the virus to replicate (Poskin et al., 2014).

While none of the viruses in the Simbu serocomplex (further subgrouping based on complement fixation test) such as: Simbu, Akabane, Shamonda, Aino, and Sathuperi has been demonstrated to cause disease in humans, the wider Simbu serogroup includes Oropouche and Iquito viruses, which cause a severe febrile syndrome in humans in South America (Aguilar et al.,

2011). As the most closely related viruses do not cause disease in humans, most authorities have concluded that the likelihood of SBV being zoonotic is minimal. The Robert Koch Institute in Germany issued a questionnaire to sheep farmers in North Rhine Westphalia which raised no suspicion of human disease as a result of SBV and all farmers were seronegative for antibodies against SBV as monitored by an immunofluorescence antibody test or virus neutralization assay. Furthermore, no viral RNA was detected when sera from these farmers were tested using RT-qPCR (European Centre for Disease Prevention and Control, 2012). A similar serological survey of 301 farmers and veterinarians with known exposure to SBV-affected herds in The Netherlands, using a VN assay, also found no antibodies against SBV (European Centre for Disease Prevention and Control, 2012), leading The European Centre for Disease Prevention and Control to declare the zoonotic risk of SBV as ‘very unlikely’.

Table 1.1 Mammalian species susceptible to SBV and classified following possible ways of identification of viral infection for each of them.

Species	Clinical signs	Direct pathogen detection (SBV genome)	Indirect pathogen detection (anti-SBV antibodies)
Alpaca		x	x
Buffalo		x	x
Bison		x	x
Camelid			x
Cattle	x (A and N)	x	x
Chamois			x
Dog		x	x
Elk		x	x
Fallow deer			x
Goat	x (rare)	x	x
Horse			x
Mouflon		x	x
Muntjac			x
Red deer			x
Roe deer			x
Sheep	x (N)	x	x
Sika deer			x
Wild boar			x

Abbreviations: A, adult animals; N, neonates (Adapted from Claine et al. (2015))

1.10.5 Clinical and pathological findings

SBV infection in adult ruminants is frequently associated with no specific clinical sign. It corresponds to a mild and transient disease, including reduced milk production (up to 50%), in appetence, hyperthermia, and diarrhea in cows (Sedda & Rogers, 2013). Only a small number of

herds had reported acute cases in adults (6% for cattle, 1% for goats, and 3% for sheep) (Bayrou et al., 2014). In December 2011, the Netherlands reported the teratogenic effect of SBV infection in sheep with clinical manifestations comparable to those observed for Akabane and Aino viruses (Kurogi et al., 1975; Tsuda et al., 2004; De Regge et al., 2013). Various congenital disorders were detected in aborted fetuses or stillborn ruminants (ovine, caprine, and bovine). Musculoskeletal deformities were commonly observed on the hind- and forelimbs, the vertebral column, and the neck. They consisted essentially of arthrogryposis, lordosis, scoliosis, torticollis, and brachygnathia inferior (Herder et al., 2012). Even if these lesions could be associated with other factor, sacral spina bifida and cleft palate were observed in 2013 in two SBV-positive stillborn lambs. In case of twin gestation, one twin may present previously described malformations, while the other only showed neurological disorders or did not present any clinical sign (Doceul et al., 2013).

Macroscopically, common malformations of the central nervous system (CNS) in young ruminants observed during necropsy were hydranencephaly, porencephaly, hydrocephalus, cerebellar and cerebral hypoplasia, and micromyelia. Histological lesions included lymphohistiocytic meningoencephalomyelitis in some cases, glial nodules mainly in the mesencephalon and hippocampus of lambs and goats, and neuronal degeneration and necrosis mainly in the brain stem of calves. Myofibrillar hypoplasia could be diagnosed in lambs and calves (Herder et al., 2012). It was suggested that lesions observed on the spinal cord were responsible for fetal immobility promoting arthrogryposis (Bayrou et al., 2014). The presence of musculoskeletal lesions due to malformations of the CNS in SBV-infected aborted fetuses or neonates led to AG-HE syndrome (Claine et al., 2015).

1.10.6 Diagnosis

SBV infection is mostly subclinical in adult ruminants or characterized, especially in cattle, by a febrile syndrome (Hoffmann et al., 2012). However, in case of transplacental infection, congenital CNS and musculoskeletal malformations such as AG-HE syndrome, aplasia or hypoplasia of the cerebrum or cerebellum, and hydranencephaly presented by aborted fetuses, stillborn, or newborn ruminants are more relevant. However, due to similarities between clinical features of SBV and other ruminant-specific viruses, only laboratory virological and/or serological diagnosis may confirm hypothesis of SBV episode (Claine et al., 2015).

The direct diagnosis of SBV infection can be realized by performing real-time quantitative polymerase chain reaction (RT-qPCR) developed by FLI in 2011 on S segment of the SBV genome (Bilk et al., 2012). Various organs were tested by RT-PCR, but only some of them are suitable for SBV detection (Bilk et al., 2012; van der Poel, 2012). It was established that cerebrum, spinal cord, external placental fluid, and umbilical cord are the most appropriate organs to detect SBV in malformed lambs or calves (Bilk et al., 2012). The brain stem also seemed to be a suitable organ to perform direct diagnosis of SBV infection (De Regge et al., 2013). The diagnosis can also be performed by immunofluorescence and virus isolation, (Tarlinton et al., 2012) but these two methods are not routinely used. The detection of SBV proteins and RNA in paraffin sections can be performed by immunohistochemistry and in situ hybridization (Gerhauser et al., 2014).

Besides the direct detection of the virus or its genome, the detection of anti-SBV antibodies present in serum of infected animals can be performed by indirect method of SBV infection diagnosis. Virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA) have been developed as tools for serological diagnosis. If the first method appears to be time-consuming, ELISA is more rapid, less expensive, and allows testing a larger number of samples,

but the possibility of cross-reactions with other orthobunyaviruses from the Simbu serogroup might occur (Wernike et al., 2017; Oluwayelu, et al. 2018). Moreover, ELISA tests are also able to detect anti-SBV antibodies in milk (Humphries & Burr, 2012). It seemed that ELISA tests presented lower sensitivity (van der Poel et al., 2014).

1.10.7 Prevention and control

Monitoring the evolution of vector population and having surveillance of the virus frequency in the vector seem to be the most appropriate ways to predict future viral episodes. Testing of the herd immunity against SBV could be another suitable approach as well. The control of insect populations and the vaccination of cattle and sheep flocks are described as the two other prophylactic measures to decrease virus transmission, prevent infection, and virus replication (Claine F, 2015).

Vaccination is a preventive measure able to reduce the impact of SBV infection (Tarlinton et al., 2012). However, farmers in some countries have already decided not to vaccinate animals against SBV. Moreover, due to the fact that SBV has rapidly spread among almost all European countries since its emergence in 2011, the surveillance of the virus appears to be more important than to vaccinate. However, the detection of novel SBV episode in Germany and in the Netherlands in late 2014 and most recently in 2016 in Belgium may reverse this situation because SBV could circulate in flocks composed of an important number of seronegative animals. Three vaccines have been developed against SBV: Bovilis SBV (MSD Animal Health), Zulvac SBV (Zoetis), and SBVvax (Merial). Both NSs (single mutant) and NSs/NSm double mutant viruses were tested as modified live vaccines in cattle with a partial and complete protection, respectively (Kraatz et al., 2015). In a study of prototype killed virus vaccines, the onset of immunity in cattle and sheep was

demonstrated 3 weeks after the second of two doses given 3 weeks apart (Wernike et al., 2013). This type of vaccine appears to be effective in preventing viral replication in animals with sheep being protected when challenged 3 weeks after a single dose (Hechinger et al., 2014). Subunit and DNA vaccines using SBV Gc have been reported (Boshra et al., 2017; Wernike et al., 2017). A very recent report indicated that the SBV Gc amino terminal domain delivered by recombinant Equine Herpes Virus 1 and Modified Vaccinia Virus Ankara conferred partial and full protection, respectively (Wernike, et al., 2018).

Vaccination usually focuses on breeding animals that receive vaccination before service in order to prevent fetal infection. It appears that in cattle, long-term immunity persists at least 6 years after natural SBV infection (Wernike et al., 2018). Based on the decay of maternal antibodies, calves could efficiently be vaccinated against SBV at an age of 6 months (Elbers et al., 2014). In sheep, neutralizing antibodies against SBV were detected 4 years after the first viral infection (Claine et al., 2018), and the lambs seem to lose maternal antibodies at an age of 4 months (Claine et al., 2015).

1.10.8 Economic impact of Schmallenberg virus

The impact of the initial SBV outbreak on the overall European economy was low (European Food Safety Authority, 2012). There was considerable variation in the cost to individual farm businesses, depending on whether their mating practices resulted in the at-risk gestation period overlapping with peak midge season. The overall economic impacts of the virus have been reported (Dominguez et al., 2014; Martinelle et al., 2014; Veldhuis et al., 2014; Barrett et al., 2015) and several economic models have been produced from these data (Alarcon et al., 2014; Raboisson et al., 2014). The main impacts of the virus on farm economics are due to milk production losses, reproductive losses as a consequence of abortion and fetus deformity, the cost of purchase of

replacement stock to compensate for reproductive losses and replacement animals not sold, as well as veterinary costs and movement restrictions (Alarcon et al., 2014). One factor that the current models have not included is the impact of early reproductive losses, since firm data are not available for this parameter. The inclusion of these losses would add to the economic impact of SBV. There is a high level of variation in the impact of SBV on individual farms, ranging from negligible to over 50% of losses of new-born animals (Helmer et al., 2013). The potential impact also needs to be considered in the light of the range of production systems for ruminants in Europe, which vary from high genetic value, intensively managed, indoor housed, year round reproduction, dairy herds to extensively grazed, low stocking density, block mated in autumn, sheep flocks. Even though, at present most countries lifted the restriction of importing semen collected in SBV-affected countries; one of the main economic impacts of the initial SBV outbreak was the loss of export markets for bovine genetics (semen, embryos and breeding stock) due to the introduction of trade barriers from countries free of SBV (60% of countries trading with Europe imposed restrictions). A decline of 10–20% in trade was observed, in addition to the value of pure-bred breeding animal exports dropping by 20% from 2011 to 2012 (European Food Safety Authority, 2014). The remaining outcome of economic significance has been the finding that potentially infectious virus is shed intermittently in semen for up to 3 months after initial infection in a small number of bulls (Hoffmann et al., 2012, 2013; Ponsart et al., 2014; Schulz et al., 2014; Van Der Poel et al., 2014). This has not been reported for rams or bucks; however, only small numbers of sheep and goats have been examined compared with cattle. Sexual transmission of the virus has not been reported; however, given the importance of artificial insemination in cattle breeding in developed countries, the risk of virus introduction has resulted in trade bans or testing requirements on semen or embryos from SBV-affected areas (Hoffmann et al., 2012). In the light of the concerns

of producers over vaccination costs and benefits, and the current risk and uncertainty over future SBV outbreaks, it is worth considering recent data on the economic impacts of the virus and the cost-benefits of vaccination as a control measure. Experience with vaccination against BTV has shown that a high rate of vaccination can substantially reduce virus circulation and the economic impact of BTV (Lazutka et al., 2015). A summary of the range of the costs of the disease versus the cost of vaccination for the main production types in Europe is presented in Table 1.2. These figures indicate that vaccination would be warranted in most beef cattle herds and dairy sheep flocks; however, in other production systems, only herds in 'high risk' categories, such those with low seropositivity and management systems where gestation and pasture availability overlaps with peak midge season would accrue an overall benefit from vaccination against SBV (Baylis et al., 2010; Alarcon et al., 2014).

Table 1.2 Cost of the disease per 1000 animal vs. cost benefit of vaccination.

Production System	Cost range of disease £/1000 head	Cost benefit of vaccination	Likelihood of use
Dairy cattle	8200 to 51400	−5720 to 37480	High risk only
Beef cattle	18000 to 30650	4080 to 16730	Yes
Lamb production	4750 to 20850	−9170 to 7000	High risk only
Dairy Sheep	10340 to 29,810	3580 to 15890	Yes

Costs are shown for high risk and low risk cases and exclude labor costs.
Vaccination cost is assumed at UK £13.92 per head (£13,920 per herd).
(Adapted from Raboisson et al. (2014))

1.10.9 Potential for re-emerging of Schmallenberg virus

SBV has already followed the pattern of re-emergence shown by other arboviruses that infect ruminants, such as Akabane and Aino viruses, when certain conditions are met. In Japan, there are epidemics of infection with Aino virus every 3–6 years as naïve animals become available

(Tsuda et al., 2004; Kono et al., 2008). There are predictable annual transmission patterns and outbreaks of Akabane virus in Australia every 10–15 years, due to expansion or temporary contraction of the vector or movement of naïve animals (Kirkland, 2015). BTV serotype 8, which shares the same vector species as SBV, re-emerged in Europe after a period of absence of clinical disease of several years and declining seropositivity in the resident ruminant population (Sailleau et al., 2017). Loss of SBV immunity has already been seen in Germany, with only 20% of newborn animals having antibodies (Wernike et al., 2015). In Belgium, animals born since the initial outbreak, the numbers seroconverting have been decreasing, i.e. 58.0–65.7% in adult animals and 20.6% in heifers, resulting in a reduced prevalence of seropositivity and, presumably, immunity (Meroc et al., 2015). In the UK and Ireland, no seroconversions were detected in 2014 or 2015, indicating a presumed absence of infection in these years (Collins et al., 2016; Stokes et al., 2016). However, SBV has continued to circulate at a low levels in continental Europe, being detected in Germany (Wernike et al., 2015), Belgium (Delooz et al., 2016)[13], UK (Stokes et al., 2018) and France (Gache et al., 2016) from 2015 to 2018. This suggests that a possible periodic resurgence of the disease when a combination of the number of naïve replacement stock reaches a critical level, together with favorable conditions for the vectors.

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Chapter 2 - Virological and Serological Responses of Sheep and Cattle to Experimental Schmallenberg virus Infection

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2.1 Abstract

Schmallenberg virus (SBV) is an Orthobunyavirus in the Simbu serogroup that emerged in Germany in late 2011 and was mostly associated with a mild transient disease of sheep and cattle. SBV is transmitted by biting midges (*Culicoides* species) and causes abortions, stillbirths and congenital defects in naïve pregnant ruminants. Two separate studies were conducted with a primary objective of better understanding the virological and serological responses of sheep and cattle to different SBV isolates after experimental infection. The second objective was to produce immunoreagents and challenge materials for use in future vaccine and diagnostics research. These studies were carried out using the following infectious inocula: (i) infectious serum (ii) cell culture-grown virus, and (iii) infectious lamb brain homogenate. The responses were assessed in both species throughout the course of the experiment. SBV RNA in serum (RNAemia) was detected as early as 2 (in sheep) and 3 (in cattle) days post-infection (dpi) and peaked on 3 and 4 dpi in cattle and sheep, respectively. Cattle had higher levels of RNAemia compared to sheep. Experimental infection with infectious serum resulted in the highest level of RNAemia in both species followed by cell culture-grown virus. A delayed, low level RNAemia was detected in cattle inoculated with infectious sheep brain. Isolation of SBV was only possible from 4 dpi sera from all cattle inoculated with infectious serum and one sheep inoculated with cell culture-derived virus. SBV neutralizing antibodies were first detected on 14 dpi in both species. No specific gross and microscopic lesions were observed in either study. In conclusion, these studies highlight not only the difference in viremia and anti-SBV antibody level against the different SBV isolates, but also the extent of the response in the two host species.

2.2 Introduction

According to the recently implemented changes by the International Committee on Taxonomy of Viruses, Schmallenberg virus (SBV) belongs to the *Orthobunyavirus* genus and family *Peribunyaviridae* (Adams et al. 2017). SBV is transmitted by midges (*Culicoides* species), and infection of ruminants leads to only transient disease in adult cattle, but abortions and congenital deformities in calves, lambs, and goat kids (Wernike et al. 2012, 2013b; Zientara and Ponsart 2014). The only confirmatory test for SBV infection is the detection of viral RNA in serum during the first week post-infection or in tissues for longer times post-infection (Wernike et al. 2012). After its initial emergence in 2011/2012 in Germany and the Netherlands (Hoffmann et al. 2012; Elbers et al. 2012), SBV continued to circulate at low levels in 2013 in previously and newly infected countries (Dominguez et al. 2014; Veldhuis et al. 2015; Wernike et al. 2014). In 2014, SBV reemerged in Germany primarily in areas less affected previously (Wernike et al. 2015). In 2015, the virus circulated at a lower level but spread throughout most of Europe, as far as eastern Russia and to the south of Greece (Claine et al. 2015). Extensive recirculation of SBV since its initial emergence was reported in 2016 in Belgium, in 2017 in France and other regions (Sohier et al. 2016; Delooz et al. 2017; Wernike et al. 2017; Gache et al. 2018).

Though, there are strong regulatory and control mechanisms to prevent the introduction of foreign animal diseases into the US, the extensive trade and travel between the US and Europe as well as the presence of both an SBV susceptible ruminant population and competent vectors (Smith et al. 1996) make Schmallenberg disease a potential threat to US agriculture. Therefore, development of detection methods and vaccines for the control and prevention of SBV infection would provide animal health practitioners with important tools to manage cases of potential SBV introduction. Until now, subcutaneous inoculation of cattle, sheep or goats with infectious serum

or cell culture-grown virus followed by monitoring of clinical signs and viremia has been the model for studying SBV pathogenesis (Wernike et al. 2012, 2013b; Laloy et al. 2015). However, in these studies there have been no direct comparisons of the use of infected brain homogenate as the inoculum instead of infectious serum or cell-culture derived virus. Furthermore, viremia is measured indirectly, extrapolated from RT-qPCR Ct value. Therefore, the primary objective of these studies were to better understand the virological and serological responses of local sheep and cattle breeds to different SBV isolates after experimental infection. A secondary objective was to produce immunoreagents and challenge materials for use in future vaccine and diagnostics research.

2.3 Materials and methods

2.3.1 SBV inoculation

SBV (BH80/11-4) was isolated from the blood of a field-infected cow (10^3 pfu/mL titer) (Hoffmann et al. 2012) and kindly provided by M. Beer, FLI (Insel-Riems, Germany). This isolate was passaged once in the *Aedes albopictus* cell line C6/36 (ATCC CRL-1660) at 37°C and 5% CO_2 for 5 days to achieve a titer of 10^5 pfu/mL before animal inoculation. The isolate was called ICCS. SBV infected lamb brain (RT-PCR positive (Ct = 30), virus isolation negative) designated as ISBH was kindly provided by Falko Steinbach, Animal Health and Veterinary Laboratories Agency, (Surrey, UK). The infectious bovine serum (IS) was produced by inoculation of calves with blood samples from diseased cows of the initial outbreak series (Wernike et al. 2012) and had a titer of 10^3 pfu/mL. Sequencing revealed that the IS and ICCS inocula contained the same SBV isolate.

2.3.2 Animals and experimental design

Experiments involving animals and viruses were performed in accordance with the Federation of Animal Science Societies Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and were approved by the Kansas State University Animal Care and Use Committees and Institutional Biosafety Committees.

In the first study, 10 Charolais sheep were assigned to three groups with each group receiving one of the following: infectious serum (IS) (n = 4), infectious cell culture supernatant (ICCS) (n = 5) or phosphate buffered saline (PBS) (n = 1). In the second study, 9 Holstein calves were assigned to three treatment groups: IS (n = 4), ICCS (n = 3) and infectious sheep brain homogenate (ISBH) (n = 2). All animals were inoculated subcutaneously with 1 mL of inoculum except for the ISBH which was administered at a 3 mL dose. On 14 dpi, cattle #7 (ICCS inoculated) and #10 (ISBH inoculated) were boosted with IS, and #9 (ISBH inoculated) was boosted with ICCS. Animals were monitored for clinical signs including rectal temperature daily until 7 dpi. Blood samples were collected from both sheep and cattle on 0, 1, 2, 3, 4, 5, 6, 7, 14 and 21 dpi. Some animals were euthanized on 4 and 5 dpi based on RNAemia level and the remaining animals (2 sheep and 3 cattle) were kept until the end of the experiment (21 dpi) in order to monitor RNAemia and seroconversion. Tissues were collected during necropsy on 4, 5, and 21 dpi (Fig. 2.1).

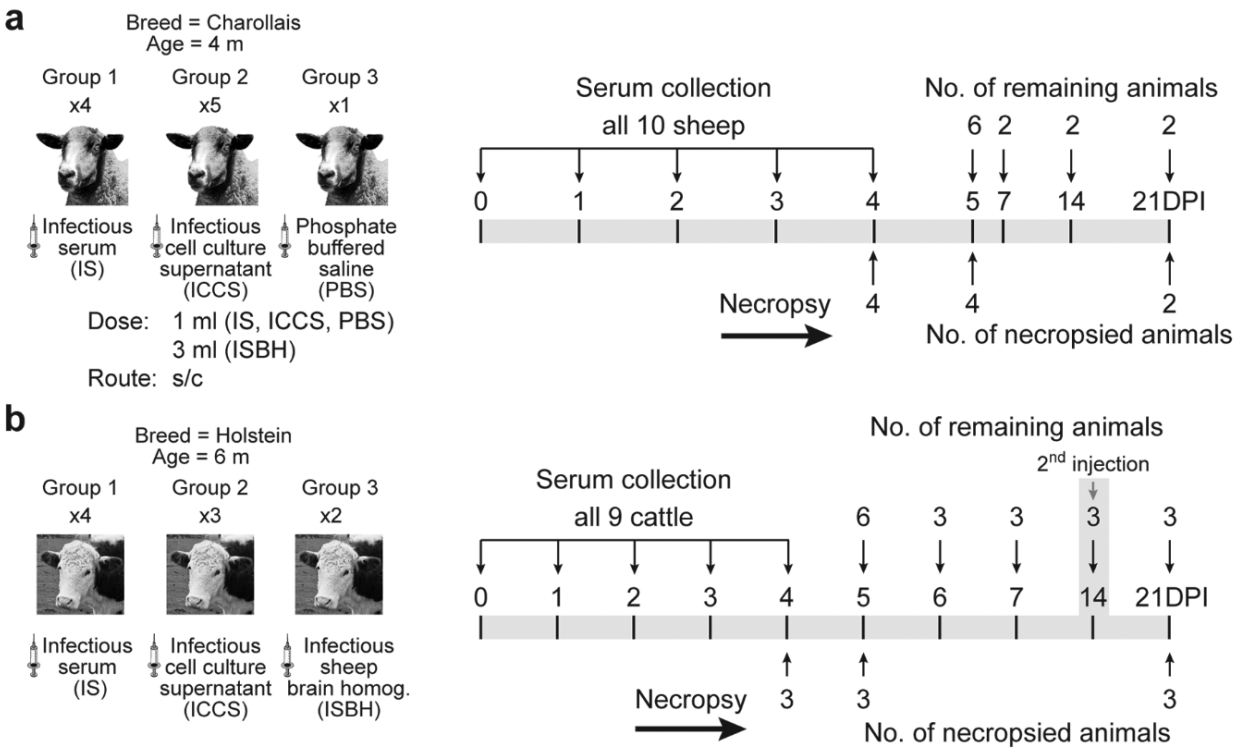

Figure 2.1 Experimental design. a) Ten sheep were assigned into three groups (G 1 received infectious serum, G 2 received infectious cell culture supernatant, & G 3 received PBS); b) Nine cattle were assigned into three groups (G 1 received infectious serum, G 2 received cell culture derived virus, & G 3 received infectious sheep brain homogenate.) The blood samples were collected on 0-7, 14, & 21 dpi.

2.3.4 Real-time RT-PCR

RNA was extracted from 250 µL of serum samples using the KingFisher automated magnetic particle processor (Thermoscientific, MA, USA) and MagMAX RNA extraction kits (Ambion Inc., TX, USA) according to the manufacturers' recommendations. The SBV genome load was determined by an S segment-specific, real-time reverse transcription PCR (RT-qPCR) as described previously (Bilk et al. 2012). The RT-qPCR assay was optimized with the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, CA, USA) using 5 µL RNA template in a total reaction volume of 25 µL and 42 cycles of amplification. A cut-off value of 35 was set based on the Ct value of the pre-SBV inoculation serum.

2.3.5 Virus isolation/titration

The viral load in serum from calves and sheep was determined by plaque assay as described previously with minor modifications (Mansfield et al. 2013). Briefly, instead of methyl cellulose, 2.4% microcrystalline cellulose Avicel RC (FMC BioPolymer, PA, USA) mixed with an equal volume of 2x minimum essential media (MEM), supplemented with 10% fetal calf serum (FCS) and 1% antibiotics was used as an overlay. Cells were fixed and stained, and the number of plaques was counted to calculate virus titer as plaque forming units per ml (pfu/mL).

2.3.6 Plaque reduction neutralization test

SBV neutralizing antibody titers in serum samples collected from cattle and sheep were measured using a plaque reduction neutralization test (PRNT) as previously described (Mansfield et al. 2013); with the minor modification mentioned above. Titers were expressed as the reciprocal of highest serum dilutions yielding ≥ 50% reduction in the number of plaques ($PRNT_{50}$).

2.3.7 Immunofluorescence assay

To further assess host serological responses, an immunofluorescence assay (IFA) was performed using the antisera obtained from the experimentally infected cattle and sheep. Vero-E6 cells were grown in 8-well chamber slides for 24 h at 37°C and 5% CO_2, or until cells reached > 90% confluency, then infected with cell culture-derived SBV with a multiplicity of infection (MOI) of 1. After 1 h incubation at 37°C and 5% CO_2, inoculum was aspirated and replaced by 1 mL of Dulbecco's minimum essential media (DMEM) with 10% FCS. Slides were incubated at 37°C and 5% CO_2 for 24 h, then the media removed and wells washed once with 1x PBS. The cells were fixed with 10% neutral buffered formalin for 15 min followed by three PBS washes,

permeabilized with 0.1% Triton-X100 diluted in 1x PBS for 5 min followed by two PBS washes and blocked for 1 h at RT with 3% normal goat serum. Anti-SBV polyclonal antibody from 21 dpi sheep and cattle, diluted 1:50 in 1x PBS, was added and the slides incubated for 1 h at 37°C and 5% CO_2. After washing the slides three times with 1x PBS, goat anti-bovine IgG-FITC conjugate (Santa Cruz Biotechnology, Inc., TX, USA) diluted to 1:200 in 1x PBS was added to each well. Slides were washed three times with 1x PBS and protected from light exposure until examined on an inverted epifluorescence microscope (Nikon eclipse TE2000-S, NY, USA).

2.3.8 Western blot assay

The serological response was further evaluated by western blot assay. SBV antigen was prepared by growing the virus in Vero-E6 cells infected with a MOI of 1. The cell lysate was then centrifuged at 13000X g for 15 min and supernatant was collected in a sterile tube and stored at -80°C until further analysis. The proteins were separated using a 12% Bis-Tris gel (Life Technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with 0.05% Tween-20 in 1x PBS (pH 7.4) containing 3% BSA for 1 h, then incubated overnight at 4°C with the study-produced bovine anti-SBV hyperimmune serum (Calf #7, 21 dpi) at a 1:50 dilution in blocking buffer. After washing the membrane with 1x PBS, goat anti-bovine IgG (H+L)-HRP conjugate (Thermoscientific, IL, USA) diluted 1:2000 in blocking buffer was applied for 1 h at 37°C. Following washing the membrane with 1x PBS, antibody binding was visualized with a 4-chloro-1-naphthol and H_2O_2 (Fluka, WI, USA) reaction.

2.3.9 Enzyme-linked immunosorbent assay

In order to measure the anti-SBV antibody response in sheep and cattle, an indirect enzyme linked immunosorbent assay (ELISA) based on baculovirus-expressed SBV nucleoprotein was used according to a previously described protocol (Breard et al. 2013; Faburay et al. 2014). The cut-off point was determined by the addition of three standard deviations to the corresponding mean OD value of the pre-vaccination serum. Mean OD values equal to or greater than the cutoff value were considered positive.

2.3.10 Statistical analysis

For statistical analysis of the change in antibody response in individual animals, un-paired t-test with Welch's correction was used in GraphPad Prism version 8.0 (GraphPad Software).

2.4 Results

2.4.1 Clinical observation

No clinical signs nor increased rectal temperatures were observed in sheep. A single calf that was inoculated with the infectious serum was pyrexic, 103.1 °F and 103.4 °F, on 3 and 4 dpi, respectively. All other infected calves had normal rectal temperatures ranging from 100.4 °F to 102.8 °F. No additional clinical responses associated with the experimental infections were noted.

2.4.2 Viremia/RNAemia level

Viral RNA was first detected in blood by RT-qPCR on 2 dpi in sheep and on 3 dpi in cattle, and was detectable until 5 and 7 dpi in sheep and cattle, respectively (Fig. 2.2). The level of RNAemia peaked on 3 and 4 dpi in cattle and sheep, respectively (Fig. 2.2). Based on absolute Ct values, animals inoculated with the infectious serum had higher levels of RNA compared to those

inoculated with cell culture-grown virus, which in turn had higher levels compared to those inoculated with the infectious brain homogenate. Additionally, the onset of RNAemia in calves inoculated with the infectious brain homogenate was delayed by 2 days. Despite daily attempts from 1-7 dpi with all cattle and sheep serum samples, virus was only isolated from 4 dpi sera from the four infectious serum inoculated calves, and one sheep infected with cell culture-derived virus (Table 2.1). Calf #5 had the highest RNAemia level with a Ct of 22 and the highest isolated virus titer of 1.8 x 10^4 pfu/mL (Table 2.1). The SBV genome was also detected in various tissues by RT-qPCR in both species (Table 2.2). Liver from 5 out of 10 sheep, and 4 out of 9 of the calves were found RT-qPCR positive on day 4 and 5 post-infection. RNA positive brain tissues were in 7 out of 10 sheep and 3 out of 8 cattle. Spleen was found positive in a significant portion of the calves (4 out of 9); however, spleen from sheep were not collected during necropsy.

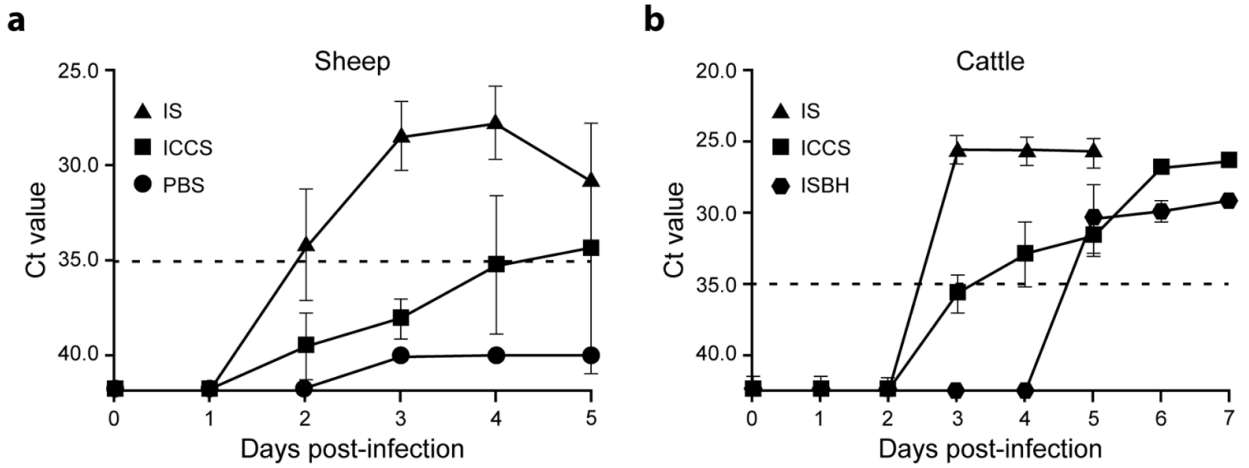

Figure 2.2 Detection of SBV RNA in serum by RT-qPCR after inoculation. (a) sheep with infectious calf serum (S 1, S 2, S 3, S 4, & S 5), infectious cell culture media (S 6, S 7, S 8, & S 9) and PBS (S 10); (b) calves with infectious calf serum (C 1, C 2, C 4, & C 5), infectious cell culture supernatant (C 6, C 7, C 8,), and infectious sheep brain homogenate (C 9, C 10). *Ct* cut-off value = 35. Data represent the average values ± SEM of each group on the days indicated.

Table 2.1 SBV titer in 4 dpi sera in sheep and cattle as determined by plaque assay.

Sheep ID	*Inoculum*	*pfu/ml*	*Cattle ID*	*Inoculum*	*pfu/ml*
S1	IS	0	C1	IS	8
S2	IS	0	C2	IS	$2x10^3$
S3	IS	0	C4	IS	$2x10^3$
S4	IS	0	C5	IS	$1.8x10^4$
S5	ICCS	0	C6	ICCS	0
S6	ICCS	0	C7	ICCS	0
S7	ICCS	0	C8	ICCS	0
S8	ICCS	0	C9	ISBH	0
S9	ICCS	8	C10	ISBH	0
S10	PBS	0			

S = sheep; C = cattle; IS = infectious serum; ICCS = infectious cell culture supernatant; PBS = phosphate buffered saline; ISBH = infectious sheep brain homogenate.

Table 2.2 Real-time RT-PCR results for tissue samples taken at necropsy.

	Animal ID																		
	S1	*S2*	*S3*	*S4*	*S5*	*S6*	*S7*	*S8*	*S9*	*S10*	*C1*	*C2*	*C4*	*C5*	*C6*	*C7*	*C8*	*C9*	*C10*
Inoculum	IS	IS	IS	IS	ICCS	ICCS	ICCS	ICCS	ICCS	PBS	IS	IS	IS	IS	ICCS	ICCS	ICCS	ISBH	ISBH
Necropsy (dpi)	4	4	5	5	5	4	4	5	21	21	5	4	4	4	5	21	5	21	21
Cerebrum	31.6	30.0	25.8	33.0	24.0	33.3	ND	ND	33.6	ND	ND	33.3	ND	27.8	33.0	ND	ND	ND	ND
Liver	29.0	29.1	28.5	ND	30.4	ND	20.3	ND	ND	ND	ND	ND	23.8	26.0	ND	ND	33.6	ND	33.2
Spleen	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	ND	29.6	23.4	22.1	ND	33.5	ND	ND	ND

IS = Infectious serum; ICCS = Infectious cell culture supernatant; PBS = Phosphate buffered saline; ISBH = Infectious sheep brain homogenate; ND = not detected; NC = tissue not collected; S = sheep; C = cattle

2.4.3 Serology

The antibody response in sheep was measured by a SBV nucleoprotein-based in-house indirect ELISA, which had high background across all samples including baseline controls. Despite this, a slight antibody level increase was visible at 21 dpi (Fig. 2.3a). In contrast, the calf sera had minimal background and a 5-fold higher response was observed at 21 dpi compared to 0 dpi for calf #7 which received cell culture-grown virus; whereas calves #9 and #10 both inoculated with SBV-positive brain homogenate failed to seroconvert (Fig. 2.3bB). Sheep #9 exhibited a

lower neutralizing antibody response, as measured by $PRNT_{50}$, with a titer of 32 at 21 dpi. The rest were negative (Table 2.3a). In contrast, neutralizing antibody titers of 1024, 16 and 256 were detected in the 21 dpi serum from calf #7, #9 and #10, respectively (Table 2.3b). Neutralizing antibodies in cattle were detected only in serum collected after booster injection at 14 dpi, since one of the objectives of this study was to produce serological test reagents that could be used in subsequent studies. Only the cattle serum was able to detect SBV infection on infected Vero cells by immunofluorescence assay. A diffused fluorescence was observed in the cytoplasm of infected cells but not in un-infected cell controls (Fig. 2.4a). Similarly, infected cell lysates were analyzed by western blot assay and the two major SBV proteins, nucleocapsid (N) and glycoprotein C (Gc) were detected at 48 h post-infection (Fig. 2.4b). Both, the recombinant N and Gc protein were detected by the day 21 cattle serum (Fig. 2.4c, d).

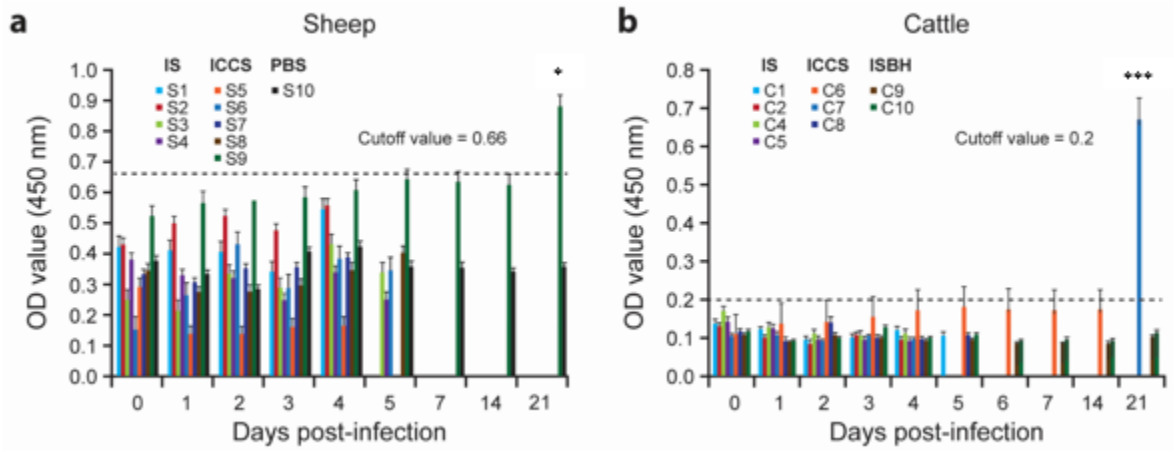

Figure 2.3 An indirect SBV antibody ELISA result. (a) Sheep after inoculation with infectious calf serum or infectious cell culture supernatant or mock. Sheep # 9 and 10 were kept until the end of the experiment (21 dpi); (b) calves after inoculation with infectious calf serum or infectious cell culture supernatant or infectious sheep brain homogenate. Calves # 7, 9 and 10 were kept until the end of the experiment (21 dpi). Statistical analysis was performed using the un-paired t-test with Welch's correction (significant difference ***$p < 0.001$; *$p < 0.05$).

Table 2.3 SBV neutralizing antibody titers in sera at different days post-infection. Results are presented as the $PRNT_{80}$ titer for sheep (a) and cattle (b) on the indicted days post-infection.

a

Sheep ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
DPI	4	4	5	5	5	4	4	5	21	21
Titer	0	0	0	0	0	0	0	0	32	0

b

Cattle ID	C1	C2	C4	C5	C6	C7	C8	C9	C10
DPI	5	4	4	4	5	21	5	21	21
Titer	0	0	0	0	0	1024	0	16	256

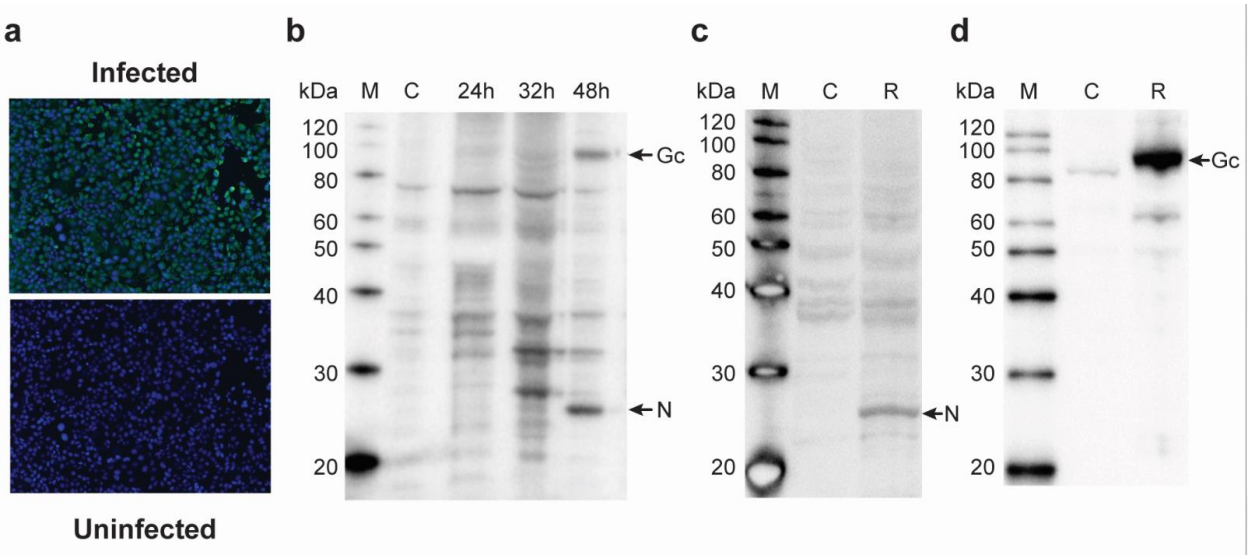

Figure 2.4 Detection of SBV on infected Vero cells. a) After 24 h using immunofluorescence microscopy; b) detection of SBV Gc & N proteins in cell lysate after 24, 32, & 48 h post-infection; c) and d) Recombinant SBV N and Gc proteins were analyzed to confirm the size of the respective proteins. M -molecular weight marker; C - cell control.

2.4.4 Pathology

Despite extensive review of the tissues collected from all animals in both studies, no lesions directly attributable to SBV were observed. However, in two calves, there were some CNS lesions that were non-specific for a variety of viruses, or other pathogens. In the brain stem of calf #4 there was a focal area of gliosis and another region with hypercellularity. Additionally, calves #6 and #7 both had rare foci of cerebral perivascular cuffing with mononuclear cells but no evidence of vasculitis.

2.5 Discussion

The experimental infection with SBV, regardless of the type of inoculum, didn't cause any observable clinical signs in either of the animal species evaluated. One calf had pyrexia on 3 and 4 dpi. Similarly, with the exception of 2 calves that had low numbers of focal changes in their brain parenchyma that could be attributable to a viral etiology, there were no histopathological

findings attributable to SBV. Immunohistochemistry for viral antigen was attempted on these tissue sets but was unsuccessful, likely due to the absence or low levels of viral antigens present post formalin-fixation, processing and paraffin-embedding. These results are consistent with Wernike et al. (2012, 2013b) as well as Poskin et al. (2014) who observed a short RNAemia in both species and no clinical signs, except diarrhea in individual animals, and gross lesions.

The onset of RNAemia after experimental infection was found to be dependent on the inoculum type. RNAemia was detected 2 days earlier in both the infectious serum and infectious cell culture supernatant inoculated calves compared to animals inoculated with the infectious brain homogenate. Wernike et al. (2013) did not detect RNA in the serum from sheep that were inoculated with virus (10^8 $TCID_{50}$/mL) originating from lamb's brain and passaged three times in Vero cells before use. The detection of RNA in the serum from the infectious sheep brain homogenate inoculated cattle, in this study, could be explained by either the species difference (cattle vs. sheep) or the inoculum itself, since in this experiment virus from lamb brain homogenate was used without prior cell culture passage. Overall, the infectious serum inoculation resulted in higher RNAemia compared to the infectious cell culture supernatant or the infectious brain homogenate in cattle as seen previously (Wernike et al. 2013b). Additionally, detection of viral RNA in the spleen, liver, and brain samples was consistent with previous reports (Wernike et al. 2012, 2013b).

Wernike et al. (2013b) detected anti-SBV antibodies on 10, 14, and 22 dpi in sheep using a commercial ELISA kit. Using an in-house indirect ELISA, we detected higher OD levels in cattle versus sheep sera when testing antibodies reacting to the SBV N protein by 21 dpi (Fig. 2.3). The same was true for the neutralizing antibody titer as measured by PRNT (Table 2.3). The higher ELISA and neutralizing antibody titers seen in the two cattle sera are most likely due to these cattle

receiving a second inoculation. The high background observed in the sheep sera ELISA may be due to an inherent species effect or prior exposure to other orthobunyaviruses. The N protein is the most conserved protein among orthobunyaviruses and elicits a strong humoral immune response in infected animals (Gonzalez-Scarano et al. 1982; Wernike et al. 2014). A recent cross-sectional study revealed a Cache valley virus (CVV) sero-prevalence rate of 49%, 17%, and 10% in sheep in the eastern, central and western United States, respectively (Meyers et al. 2015). We ruled out CVV by performing PRNT with the collected sheep sera which showed absence of neutralizing antibodies except sheep #4 (data not shown). However, the high background observed in sheep could be due to the circulation of other unidentified orthobunyaviruses in the sheep population of the region.

The increase in the antibody response in cattle (Fig. 2.3b) after boost with the infectious serum seems to be dependent on the initial type of inoculum given to the cattle. This increase was observed in the calf primed with the cell culture-derived virus, but not in the calves inoculated with brain homogenate. Since no animal from the infectious serum inoculated cattle group were kept past 5 dpi, we do not know the response in this group. The hyper-immune serum collected from a calf on 21 dpi was used to detect SBV replication in cell culture by IFA which is more specific than cytopathic effect (Fig. 2.4a). Similarly, the same serum detected both the SBV Gc and N proteins on the western blot indicating its suitability as a reagent for detection of SBV antigens (Fig. 2.4b). The size of the two proteins was confirmed with separate western blots using recombinant SBV N (Fig. 2.4c) and Gc (Fig. 2.4d) proteins.

2.6 Conclusion

SBV has spread throughout most of Europe since its initial detection in 2011 in Germany. However, to date, this virus' emergence in Europe is poorly understood (Claine et al. 2015). Consequently, SBV poses a serious threat to other countries like the US, which has competent

insect vector species (Smith et al. 1996), susceptible livestock populations and conducts extensive trade with Europe. Although, both cattle and sheep are reported as good experimental animals for researching SBV disease pathogenesis and vaccine efficacy, to our knowledge there is no published information on the response and pathogenesis of SBV infection in local US sheep and cattle breeds. These studies highlighted not only the difference in viremia and anti-SBV antibody level against the different SBV isolates, but also the extent of the response in the two host species. The virological and serological responses were more apparent in cattle than in sheep. Overall this work creates an important basis for subsequent development and evaluation of effective countermeasures such as novel vaccines and improved diagnostics.

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Chapter 3 - Immunogenicity and Efficacy of Schmallenberg Virus Envelope Glycoprotein Subunit Vaccines

The data in this section has been submitted to the Journal of Veterinary Science.

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3.1 Abstract

Schmallenberg virus (SBV) is an orthobunyavirus of the Simbu serogroup that causes abortions, stillbirths and congenital defects in naïve pregnant sheep and cattle. Inactivated, live attenuated, subunit, DNA-mediated and live-vectored vaccines have been developed in endemic countries. There is interest in the development of SBV vaccines that would allow differentiation of infected from vaccinated animals (DIVA strategy). Few DIVA-compatible vaccines have been already developed. Similarly, this study was conducted to develop DIVA-compatible SBV vaccines using SBV glycoproteins in a different expression platform, baculovirus. The first trial included 2 groups administered either Gc (100 µg/dose) or phosphate buffered saline (PBS) and booster vaccination 2 weeks after the first vaccination. In the second trial, 3 groups were administered either Gc (150 µg/dose), Gc and Gn (150 µg of each) or PBS. Booster vaccination after 3 weeks. Animals were challenged 9 days and 3 weeks after booster vaccination in the first and second studies, respectively with bovine serum containing 10^4 pfu of SBV. All vaccines and PBS controls were prepared with adjuvant and administered subcutaneously. Using an SBV Gc-specific ELISA, antibodies were first detected in serum samples at 14 days after the first vaccination in both trials and peaked on days 7 and 9 after the booster in the first and second trial, respectively. Low titers of neutralizing antibodies were detected in serum from only 3 out of 6 and 2 out of 4 animals in the first and second trial, respectively at 14 days after the first vaccination. The titers increased 2 to 3-fold after booster vaccination. However, SBV-specific RNA was detected in the serum and selective tissues in all animals after challenge. Neither of the SBV candidate vaccines prevented viremia nor conferred protection against SBV infection.

3.2 Introduction

Schmallenberg virus (SBV) is a member of the *Peribunyaviridae* family, *Orthobunyavirus* genus and *Schmallenberg orthobunyavirus* species (Adam et al., 2017). Discovered in Germany in 2011 (Hoffmann et al., 2012; Wernike et al., 2012), it has since spread throughout the European continent (Yilmaz et al., 2014). The disease primarily affects ruminants including cattle, sheep and goats. SBV infection has been associated with diarrhea, fever and decreased milk production (Hoffmann et al., 2012; Lievaart-Peterson et al., 2015; Peperkamp et al., 2015; Wernike et al., 2013). SBV infection has been linked to widespread abortions and developmental malformations in newborn domestic ruminants (Bayrou et al., 2014). SBV is an arthropod-borne pathogen that is transmitted by biting midges (*Culicoides* spp.) (Larska et al., 2013). Inactivated vaccines have been developed and are commercially available for the prevention of economic losses associated with SBV infections (Hechinger et al., 2014; Kraatz et al., 2015; Wernike et al., 2013); however, those vaccines do not offer the ability to differentiate infected from vaccinated animals (DIVA compatibility). Commercial nucleocapsid (N)-based ELISA tests are available for SBV antibody detection (Bréard et al., 2013; Humphries et al., 2012) and have been used extensively for surveillance.

The SBV genome, similar to other orthobunyaviruses, has a tripartite single-stranded RNA genome of negative polarity consisting of small (S), medium (M) and large (L) RNA segments. The S segment encodes the N protein and the nonstructural protein NSs. The M segment encodes two structural glycoproteins, amino-terminus glycoprotein (Gn) and carboxyl-terminus glycoprotein (Gc), and the nonstructural protein NSm. The L segment encodes an RNA-dependent RNA polymerase (Elliot et al., 2013; 2014). The two orthobunyavirus glycoproteins differ in size: Gn is 32–35 kDa, whereas Gc is 100–110 kDa. Both proteins are type I integral membrane proteins

modified by *N*-linked glycosylation and are rich in cysteine residue. The humoral immune response against mammalian cell-expressed SBV glycoproteins has been analyzed and only Gc but not Gn protein was recognized by SBV hyperimmune sera in various serological assays (Roman-Sosa et al., 2016).

Our lab previously reported a recombinant subunit vaccine for Rift Valley Fever (RVF) using baculovirus expressed RVFV glycoproteins which resulted in efficient protection from virulent challenge (Faburay et al., 2014). Similarly, amino acid domains associated with virus neutralization have recently been identified in the M-segment, specifically on amino domain within the SBV Gc (Hellert et al., 2019). Previously, different subunit vaccines containing the full ectodomains of both Gc and Gn, and the critical amino acid domain of Gc (expressed in mammalian cells) or as DNA plasmid vaccines were tested in cattle and IFNR $^{-/-}$ mice resulting in partial protection (not all vaccinated animals were protected from developing viremia in cattle or from mortality in mice experiments) (Boshra et al., 2017; Wernike et al., 2017). Recombinant Equine Herpes Virus 1 and Modified Vaccinia Virus Ankara expressing amino acid domain of SBV Gc conferred partial and full protection, respectively (Wernike, et al., 2018). Based on our previous success in developing a subunit vaccine for RVFV, we expressed the ectodomains of SBV Gc and Gn in baculovirus and used these proteins as candidate vaccines to immunize cattle in a prime-boost approach. Immunogenicity, induction of neutralizing antibodies and subsequent prevention of viremia and tissue-associated virus were assessed after challenge.

3.3 Materials and methods

3.3.1 Construction of recombinant baculovirus expressing SBV Gn and Gc

The cloning and construction of the recombinant baculovirus was carried out similar as described previously (Faburay et al., 2014). Briefly, the coding sequences of the SBV structural proteins Gn and Gc were obtained from the SBV BH80/11-4 isolate (GenBank accession no. HE649913.1), and were synthesized (GENEWIZ, Inc., San Diego, CA, USA) with the following molecular modifications. For both proteins, the transmembrane domain and cytoplasmic tail were deleted from the coding sequence and a C-terminal 6xHistag was added to facilitate protein purification by affinity chromatography using Ni-NTA superflow resin (Novagen, Rockland, MA, USA).

3.3.2 Expression and purification of SBV Gn and Gc proteins

Purified recombinant bacmids carrying the respective coding sequences of SBV Gn and Gc were transfected using Cellfectin II Reagent (Invitrogen–Life Technologies) into Sf9 cells (Invitrogen–Life Technologies), grown in Sf-900 II SFM medium (Invitrogen–Life Technologies) and supplemented with 10% FBS and 100 U/mL penicillin-streptomycin. Protein expression was carried out using P4 passage recombinant baculovirus stock [> 10^7 plaque-forming units (pfu)/mL]. Western blot analysis of the lysate was performed to confirm specific protein expression as described below. The recombinant proteins were purified using Ni-NTA Super flow resin according to the protocol described previously (Faburay et al., 2013). The protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermofisher Scientific, IL) at an absorbance of 562 nm, using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as the protein standard. Aliquots were stored at – 80 °C until use.

3.3.3 Western blots to detect recombinant SBV Gc and Gn proteins

The detailed procedure for detection of baculovirus-expressed recombinant proteins by western blot has been described previously (Faburay et al., 2013). Briefly, the proteins were separated using a 12% Bis-Tris gel (Life Technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with 0.05% Tween-20 in 1x PBS (pH 7.4) containing 3% BSA for 1 h, then incubated overnight at 4°C with mouse anti-histidine horseradish peroxidase (HRP) conjugated monoclonal antibodies at a dilution of 1:5000 (Thermofisher Scientific, IL). After the final washing steps with 1x PBS, specific reactivity was detected using 3-amino-9-ethyl-carbazole (AEC) peroxidase substrate (Sigma-Aldrich, St. Louis, MO) detection system.

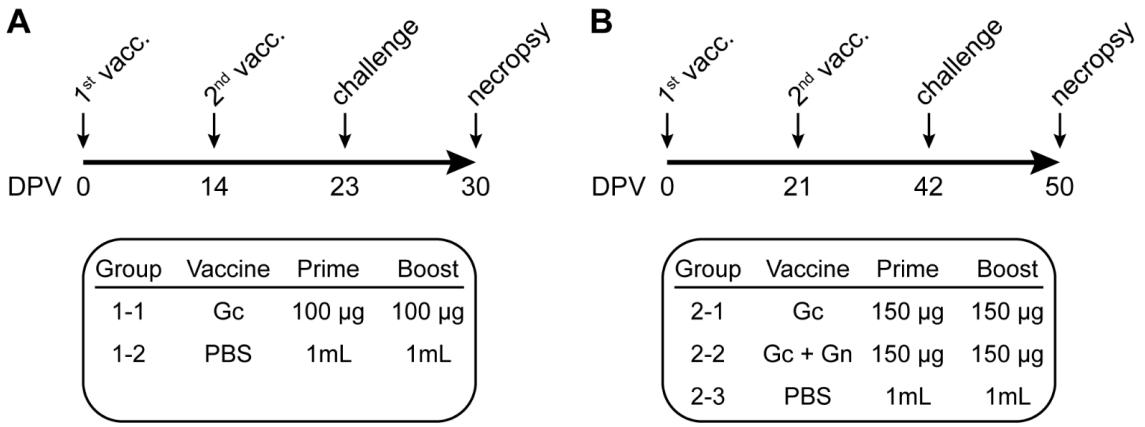
3.3.4 Vaccine preparation

The purified Gn/Gc glycoproteins were formulated according to the manufacturer's instruction in Montanide ISA 25 oil-in-water adjuvant (Seppic, France) to obtain a concentration of 100 or 150 µg of each immunogen per vaccine dose in the first and second vaccine trials, respectively. The vaccines were administered subcutaneously.

3.3.5 Animals and experimental design of challenge studies

Experiments involving animals and viruses were carried out at the Biosecurity Research Institute of Kansas State University in accordance with animal welfare guidelines and were approved by the Kansas State University's Institutional Animal Care and Use Committee (IACUC #-3575). Cattle were selected as the target animal of choice for the vaccine trials based on our previous studies that showed more apparent virological and serological responses in cattle than sheep (Endalew et al., 2018). Twenty-four, six-month-old, Holstein-Friesian cattle were purchased

locally from a commercial farm and used in two separate studies. In both studies, animals were randomly assigned to the different experimental groups using random number table. The first vaccine trial had 2 groups (n=6 each) of cattle. Group 1 was administered Gc (100 µg/dose) and group 2 PBS. Animals were boosted with the same dose 2 weeks after the first vaccination and challenged 9 days after the booster vaccination with 1 mL of bovine serum containing 10^4 pfu SBV strain BH80/11-4, subcutaneously (Fig. 3.1a). The second vaccine trial had three groups (n=4 each) of cattle; group 1 was administered Gc (150 µg/dose), group 2 Gc and Gn combined (150 µg of each/dose) and group 3 PBS. Booster injection of the same amounts of the respective antigens were given three weeks following the first vaccination. The unvaccinated control group received 1 mL of PBS mixed with adjuvant. Cattle were challenged 3 weeks after the second vaccination (Fig. 3.1). All vaccines were given subcutaneously. At the end of the experiments, all animals were humanely euthanized by intravenous administration of Fetal-Plus (Vortech Pharmaceuticals, MI, USA) at a dose of 1 mL per pound of body weight.



Group	Vaccine	Prime	Boost
1-1	Gc	100 µg	100 µg
1-2	PBS	1mL	1mL

Group	Vaccine	Prime	Boost
2-1	Gc	150 µg	150 µg
2-2	Gc + Gn	150 µg	150 µg
2-3	PBS	1mL	1mL

Figure 3.1 Experimental design. a) Trial 1, 12 calves were assigned to two groups (G1-1 received 100 µg of SBV Gc, G1-2 received PBS); b) Trial 2, 12 calves assigned to three groups (G2-1 received 150 µg of SBV Gc, G2-2 received SBV Gc/Gn (150 µg each), and G2-3 received PBS). Booster injection given after 2nd and 3rd week post-first vaccination in trial 1 and 2, respectively. Vaccines and PBS were prepared with adjuvant.

3.3.6 Blood sample collection

Blood specimens were collected from the jugular vein of all animals prior to vaccination (day 0) to establish a baseline pre-vaccination immune response status. Thereafter, serum samples were prepared from the blood collection from all cattle on 7, 14, 23 and 30 (first trial) and 14, 21, 28, 35, 42 and 49 (second trial) day post-vaccination (dpv). Sera were stored at –20°C until use. The SBV positive control serum (21 days post-infection cattle serum) was obtained from a previous study (Endalew et al., 2018).

3.3.7 Enzyme-Linked Immunosorbent Assay Response

Specific antibody response in serum was measured using an indirect enzyme-linked immunosorbent assay (ELISA) based on the baculovirus-expressed SBV Gc protein. The ELISA assay was performed according to a previously described protocol (Bréard et al., 2013; Faburay et al., 2014). The cutoff point was determined by the addition of two standard deviations to the corresponding mean OD value of the pre-vaccination serum. Mean OD values equal to or greater than the cutoff value were considered positive.

3.3.8 SBV neutralizing antibodies

SBV neutralizing antibody titers in serum samples were measured using a plaque reduction neutralization test (PRNT) as previously described (Mansfield et al., 2013) with minor modification. Briefly, 2.4% microcrystalline cellulose Avicel RC (FMC BioPolymer, PA, USA), mixed with an equal volume of 2x minimum essential media (MEM) containing 10% fetal calf serum (FCS), was used as an overlay. The stock virus (10^6 pfu/mL), grown in BHK21 cells, was diluted to result in 50 pfu/well to be used in the PRNT assay. Equal volumes of test serum and virus (125 µL each) per well in a 96-well plate were incubated at 37 °C for 1 h before transfer to

80% confluent Vero-E6 cells, grown in a 24-well plate. Ten 2-fold dilutions, each in duplicate, were tested. Negative, positive, and cell control wells were included. The neutralization titer was expressed as the reciprocal of highest serum dilutions yielding ≥ 80% reduction in the number of plaques. The results are presented as average titer (log2) of each group at different time points.

3.3.9 RNA extraction

Tissue samples (spleen, cervical, mandibular, mesenteric lymph nodes and peyer's patches) collected at necropsy were homogenized in 1 mL MEM/100 mg tissue using stainless steel beads in tissuelyser II (Qiagen Inc., CA, USA). Each aliquot of 250 µL tissue lysate was added to 750 µL of Trizol LS reagent and incubated for 5 minutes at room temperature. Collected serum was treated similarly. 100 µL of bromochlorophenol was added to the mixture in order to separate the aqueous phase. Samples were vigorously shaken for 15 seconds and incubated at RT for 5 minutes. The mixture was centrifuged at 12,000 RCF for 15 minutes at 4 °C and the aqueous layer was transferred to a clean tube. 100 µL of the aqueous layer of each sample mixed with 15 µL (10^6 copy number per microliter) exogenous internal positive control (XIPC) was used for RNA extraction using one of the following two extraction protocols. The King Fisher automated magnetic particle processor (Thermo Fisher, MA, USA) and MagMAX Viral RNA extraction kit (Ambion Inc., TX, USA) in trial 1 and the Qiagen BioSprint 96 automated magnetic particle processor (Thermo Fisher, MA, USA) and the MagAttract 96 cador pathogen kit (Qiagen Inc., CA, USA) in trial two were used according to the manufacturer's recommendation.

3.3.10 Reverse Transcriptase Real time PCR

SBV genome detection was performed using a S segment-specific quantitative real-time reverse transcription PCR (RT-qPCR) as described previously (Bilk et al., 2012) with the

following modifications. In trial 1, the RT-qPCR assay was optimized with the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, CA, USA) using a total reaction volume of 25 µL. In trial 2, amplification was carried out using qScript XLT One-Step RT-qPCR Tough Mix master mix (Quanta Biosciences, MA, USA). 5 µL of RNA template was added per 20 µL reaction volume. The thermocycling conditions were: 50 °C for 20 minutes, 95 °C for 5 minutes, followed by 95 °C for 10 seconds, and 60 °C for 1 minute for 45 cycles. Each sample was tested in triplicate and both positive and negative controls were included in each run. The cut-off cycle threshold (Ct) value was set at 34, based on the Ct value of the pre-SBV inoculation serum.

3.3.11 Statistical analysis

For statistical analysis among groups, analysis of variance (ANOVA) test was used in GraphPad Prism version 8.0 (GraphPad Software).

3.4 Results

3.4.1 Expression of recombinant SBV Gc and Gn proteins

Expression of SBV Gc and Gn proteins was confirmed by western blot assay using mouse monoclonal antibodies directed against the C-terminal histidine tags of the recombinant proteins. SBV Gc and Gn proteins had specific bands in the western blots that match the expected molecular weight of the recombinant proteins (Fig. 3.2).

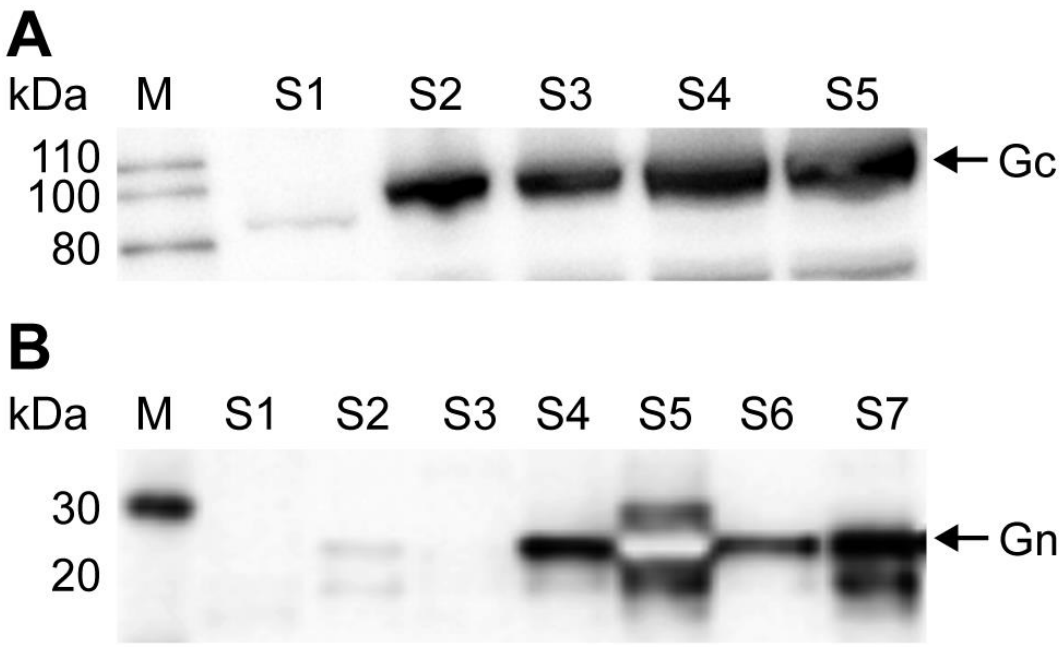


Figure 3.2 Schmallenberg virus glycoprotein expression. Recombinant SBV Gc and Gn proteins expression was confirmed by western blot assay using anti-histidine monoclonal antibodies; (a) Gc, (b) Gn. M = molecular weight marker, S = different batches of protein specimen.

3.4.2 Clinical observations after vaccination and challenge

None of the animals in trial 1 or 2 had any adverse reaction to vaccination except a mild localized swelling detected at the vaccination site, which quickly resolved and was most likely associated with the vaccine/adjuvant depot. No clinical signs attributable to SBV were observed after challenge infection, not even in the unvaccinated challenge control. Most of the cattle (9 out of 12) in trial 1 had clinical coccidiosis and were treated with sulfaquinoxaline (6 mg/lb/day for 5 days). The treatment was successful and symptoms like diarrhea and loss of appetite disappeared within several days of treatment during the vaccination phase of the study.

3.4.3 Serology

Serum was collected weekly and analyzed for SBV-specific antibodies using an indirect ELISA against the SBV Gc protein, the common component of both vaccines. SBV Gc-specific antibody response was first detected in serum samples by 14 dpv for all vaccine groups. In the first trial, the antibody level increased until virus challenge on 23 days post-vaccination (dpv) (Fig. 3.3a). In the second trial, the antibody response doubled in both Gc and Gc/Gn groups from day 14 to 28 and then decreased by 42 dpv (Fig. 3.3b). No antibody response was detected in the unvaccinated control groups before challenge infection on 23 (first trial) and 42 (second trial) dpv. An increase in Gc-specific antibody reactivity was observed in all animals a week after challenge.

To measure vaccine-induced neutralizing antibody response, a $PRNT_{80}$ was performed. SBV positive and negative sera were used as controls. In trial one, a very low ($PRNT_{80}$ titer of 1:4) of SBV-specific neutralizing antibodies was detected in 3 out of 6 animals of the Gc group at 14 dpv. The titers increased to 1:8 (2/4) and 1:16 (2/4) in 4 out of 6 animals, on the day of challenge (23 dpv). The average titer of each group in trial 1 for the different time points is presented in figure 3.4a. Similarly, in the second trial a titer of 1:4 was present in 14 dpv sera for both the Gc and Gc/Gn vaccine groups. On the day of challenge (42 dpv), titers of 1:4, 1:8, 1:8 and 1:16 for Gc group and 1:4, 1:8, 1:16 and 1:16 for the Gc/Gn group were measured. The average titer for each group in trial 2 is presented in figure 3.4b. Neutralizing antibodies were detected in all animals including non-vaccinated controls at 7 days after challenge infection.

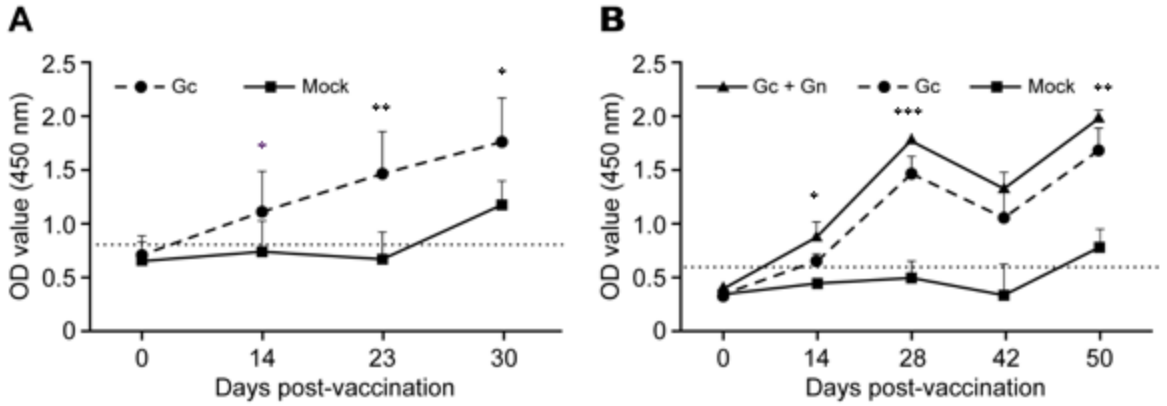


Figure 3.3 Host SBV-specific antibody responses. Results of serum samples tested by Gc-specific indirect ELISA in trial 1 (a) and 2 (b). Plates were coated with recombinant SBV Gc protein. The cutoff point (a = 0.8, b = 0.6) was determined by the addition of two standard deviations to the corresponding mean OD value of the pre-vaccination serum. Each data point represents the average values ± SEM of either 6 (a) or 4 (b) calves in each group on the days indicated. Statistical analysis was performed using the two-way ANOVA (significant difference *p<0.05; **p<0.01; ***p<0.001).

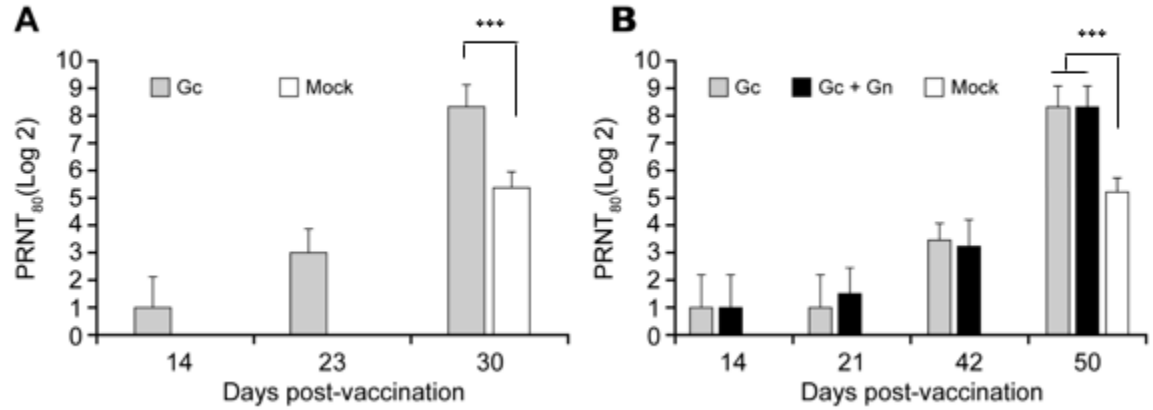


Figure 3.4 Host SBV neutralizing antibody response. Antibody titers in cattle following vaccination and challenge measured by plaque reduction neutralization test ($PRNT_{80}$). Average titer (log2) of each group in trial 1 (a) and 2 (b) at different time points post-vaccination. Statistical analysis was performed using the two-way ANOVA (significant difference ***$p <$ 0.001).

3.4.4 Viremia/RNAemia

Since SBV infection in non-pregnant animals is mostly subclinical, detection of viral RNA in serum of infected animals is the criteria for evaluation of vaccine efficacy after challenge infection (Boshra et al., 2017; Kraatz et al., 2015; Wernike et al., 2017). Therefore, RT-qPCR was performed on serum samples from 0 to 7 days post-challenge (dpc) and on tissue samples obtained at necropsy. SBV RNA was detected in serum from animals in all groups from trial 1 and trial 2 starting from 1 dpc, with a peak at 3 dpc. SBV RNA remained detectable until 5 dpc independent of vaccination status and become negative at 6 dpc (Fig. 3.5a, 3.5c). Additionally, SBV RNA was detected in all tissues collected, cervical, mandibular, mesenteric lymph nodes, spleen and peyer's patches. The mesenteric lymph node had the highest SBV RNA load (lowest Ct value) in both trials when compared to the other tissues (Fig 3.5bB, 3.5d).

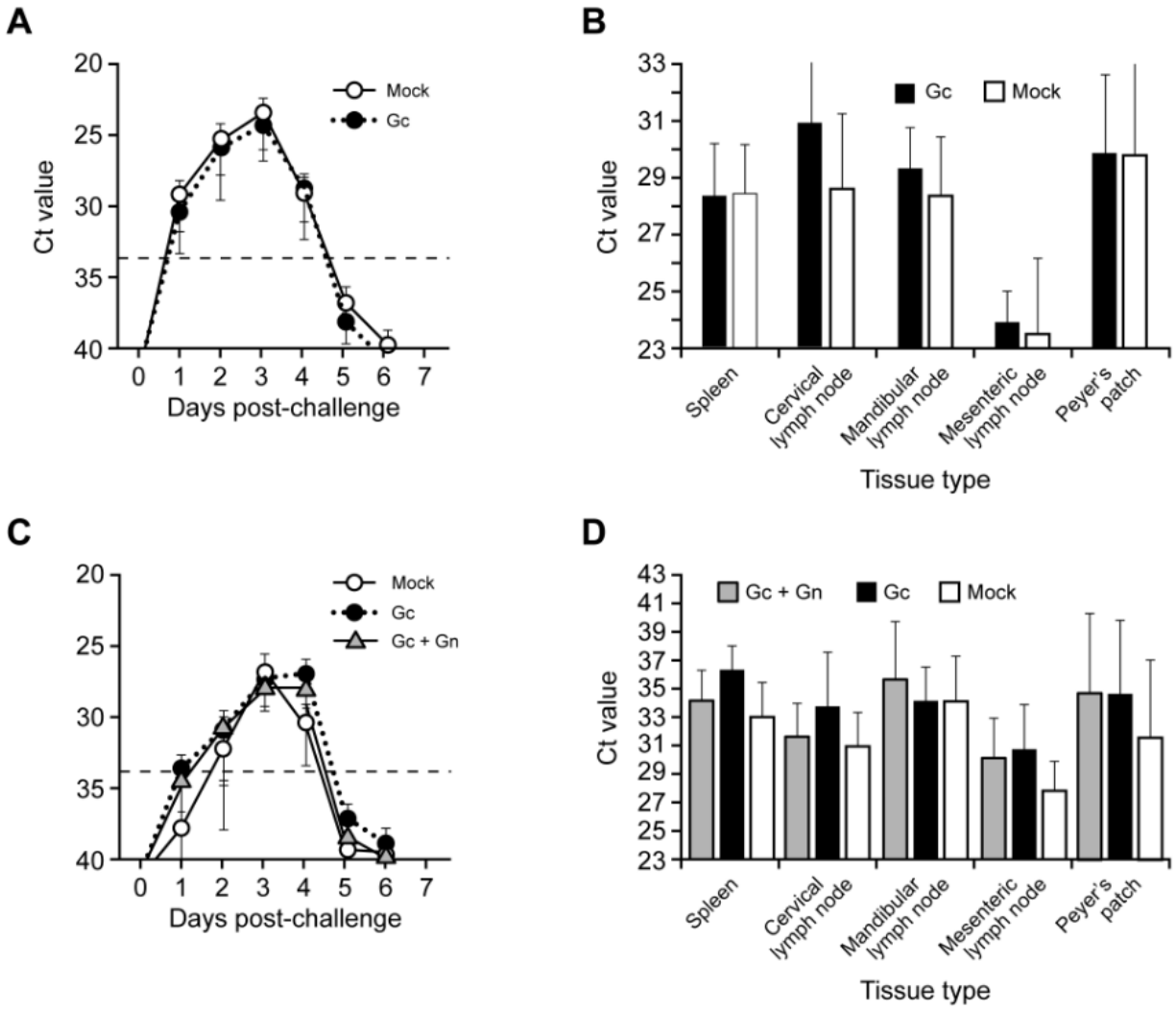


Figure 3.5 Results of the S-segment based RT-qPCR. SBV RNA detection in serum and different tissue samples from cattle vaccinated with SBV Gc, SBV Gc/Gn, or mock-vaccinated animals for trial 1 (a, b) and 2 (c, d). *Ct* cutoff value = 34. Data represent the average values ± SEM of either 6 (a, b) or 4 (c, d) calves in each group on days and tissues indicated.

3.5 Discussion

SBV vaccines based on inactivated virus formulations have been developed and effectively prevent RNAemia, clinical disease, and fetal malformation and premature birth or stillbirth (Wernike et al., 2013). Although inactivated vaccines are considered to be safe under field conditions, they usually induce weaker or shorter-lived immunity than live vaccines. Modified live virus (MLV) vaccines induce strong immune responses but have their own safety concerns which include the risk of contamination with virulent strains or other unwanted pathogens and the potential of reversion to virulence (Henderson et al., 2005; Shams et al., 2005). A recombinant modified live virus vaccine with a NSs deletion in the S segment alone and double mutant including NSm in the M segment have been tested for efficacy and conferred good protection from infection; in addition, these mutants could be DIVA compatible (Kraatz et al., 2015). The safety of this MLV vaccine is of concern since it has been shown that serial passages in cell culture restored virulence based on a single substitution acquired in the Gc protein (Varela et al., 2016). At the time this study was initiated, none of the available vaccines enabled clear differentiation of infected from vaccinated animals (DIVA). Meanwhile, this time DIVA-compatible subunit and modified live vaccines have been developed (Wernike et al., 2017, 2018; Boshra et al., 2017).

Animals vaccinated with both the Gc and Gc/Gn subunit vaccines seroconverted by 14 dpv and exhibited a time-dependent increase in antibody reactivity in both trials. This is in concordance with other reports, where vaccine preparations containing a peptide sub-domain of Gc induced time-dependent SBV-specific antibody responses (Wernike et al., 2017; Boshra et al., 2017). The development of neutralizing antibodies which block infection or viremia, a correlate of protection (Plotkin, 2010), is a key attribute for vaccines against the Simbu serogroup viruses including SBV (Wernike et al., 2013). Low titers ($PRNT_{80}$ = 8-16) of SBV-specific neutralizing antibodies were

detected in three out of six animals in the Gc group (trial 1) and two out of four animals (trial 2) in the Gc and Gc/Gn groups; however independent of the vaccination status of the animals, SBV RNA was detected in serum and tissues of these SBV-antibody positive animals by RT-qPCR, indicating the absence of protection against SBV infection. No significant difference in RNAemia between vaccinated and non-vaccinated animals was noted. This might be due to the fact that the titers of neutralizing antibodies were not sufficient to protect animals from challenge infection. A similar finding was reported by others when neutralizing antibodies elicited by the full ectodomains of the Crimean-Congo hemorrhagic fever virus Gn and Gc glycoproteins expressed in insect cells failed to confer protection against this bunyavirus (Kortekaas et al., 2015). The effect of clinical coccidiosis in the vaccine outcome in the first trial was ruled out due to similar results obtained in the second trial where the animals were free of this parasite.

Neither a DNA vaccine encoding the SBV Gc amino terminal domain (amino acid, aa. 678-947) nor a vaccine based on *E. coli* expressed SBV Gc amino terminal domain (aa 468-702) conferred protection against SBV infection in a mouse model (Wernike et al., 2017; Boshra et al., 2017). Prevention of weight loss and reduced viremia relative to the unvaccinated controls were reported for the DNA vaccine (Boshra et al., 2017). Interestingly, partial protection was observed when the SBV Gc amino terminal domain (aa 468-702) was expressed in human embryonic kidney (HEK) cells and used as a vaccine formulation (Wernike et al., 2017). Subunit vaccines containing the full ectodomains of both Gc and Gn covalently linked or subdomains of Gc have been shown to confer partial protection (one out of four animals protected); however, full protection was obtained only when the amino terminal domain of SBV Gc was linked to the corresponding domain of the related Akabane virus (Wernike et al., 2017). A very recent report indicated that the SBV

Gc amino terminal domain delivered by recombinant Equine Herpes Virus 1 and Modified Vaccinia Virus Ankara conferred partial and full protection, respectively (Wernike et al., 2018). The variations observed in the responses against the SBV subunit vaccines discussed above could be explained by the difference in the accessibility of the protective immunogen of the Gc protein used in each vaccine preparation (full ectodomain vs. amino terminal domain). Furthermore, differences in expression systems (mammalian cells vs. baculovirus vs. *E. coli* vs. virus-vectored) can affect post-transcriptional modifications and folding of the proteins, which in turn affect the immunogenicity and type of antibody response elicited by the antigen (Duan and Walther, 2015). The low neutralization antibody titers and lack of protection in the current studies using a Gc and Gc/Gn subunit vaccines are contrary to previously reported high efficacy of a Gn and Gc-based subunit vaccine for SBV (Wernike et al., 2017, Hellert et al., 2019) and another bunyavirus, Rift Valley fever (Faburay et al., 2016).

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Chapter 4 - Development and Evaluation of Diagnostics for Schmallenberg virus

4.1 Abstract

After the emergence of SBV in Europe in the fall 2011, numerous diagnostic tests for SBV genome or antibody detection have been established. These tests are essential for epidemiological investigations and diagnosis of disease outbreaks. Though the US is free of SBV disease, the extensive trade and travel between the United States and Europe along with the presence of both an SBV susceptible ruminant population and competent vectors make Schmallenberg disease a potential threat to U.S. agriculture. Therefore, the current study was performed with the following objectives: 1. Compare the performance of different SBV proteins as an antigen in an indirect antibody ELISA platform; 2. Evaluate the efficiency of three commercial nucleic acid (NA) extraction kits in extracting SBV RNA from cell culture supernatant; 3. Compare the efficiency of gene-specific RT-PCRs (S, M, L) in both single- and multiplex format.

Whole SBV lysate obtained from cell culture and three recombinant proteins [nucleocapsid (N), glycoprotein N (Gn), and glycoprotein C (Gc)] expressed in baculovirus were evaluated for suitability as ELISA antigen in detection of SBV antibodies in serum. Virus neutralization positive and negative sera generated during the SBV animal model studies were used in this experiment. The aqueous phase of trizol-inactivated, ten-fold serial dilutions of SBV from cell culture supernatant were used in evaluating the efficiency of three nucleic acid extraction protocols, namely: MagMax (Applied Biosystem), MagAttract (Qiagen), and Taco (GeneReach). Similarly, ten-fold serial dilutions of *in vitr*o transcribed SBV RNA were used to assess the efficiency of segment-specific RT-PCRs in both single- and multiplex format. Among the different SBV proteins evaluated for SBV antibody detection, both N and Gc were the most immunogenic

antigens, followed by the Gn. On the other hand, the whole SBV lysate was the least reactive in the indirect ELISA platform. The GeneReach total NA extraction kit yielded approximately 10 times more SBV RNA as compared to the Qiagen and Applied Biosystems extraction kits. Multiplex RT-qPCR for SBV resulted in good amplification of the L and M genes. A slight reduction in the amplification of the S gene in the multiplex setting was observed when compared to the singleplex assay, equating to approximately a half a log reduction in sensitivity.

The highly specific Gc-based ELISA could be the choice of a serological assay for the detection of SBV antibody in areas where Simbu serogroup viruses are endemic because the N antigen is prone to cross-reactivity. The GeneReach total NA extraction protocol is the most efficient for recovery of SBV RNA from trizol-treated cell culture supernatant. Our result also demonstrates that although the singleplex S gene assay has very good sensitivity, the multiplex detection of the S, M and L genes could enhance SBV diagnostic specificity, which is important in view of emerging novel Simbu serogroup viruses.

4.2 Introduction

Clinical diagnosis of Schmallenberg disease is difficult to make on the basis of clinical manifestations of diseases which also varies by species. Bovine adults may show a mild form of acute disease during the vector season, whereas congenital malformations occur in more species of ruminants including cattle, sheep, and goat; usually within the gestation period of the species after the vector season (Afonso et al., 2014; Bayrou et al., 2014). From suspected adults, samples of EDTA-preserved blood as well as serum should be collected and transported refrigerated to the laboratory. Samples should be collected from animals during the acute stage of clinical infection (e.g., fever, reduced milk yield, diarrhea). From aborted fetuses or deformed newborn animals, samples should be collected for histopathological, serological and virological examinations as appropriate. Tissue samples of brain, preferably cerebrum, cerebellum and brainstem along with spleen and blood are recommended (Bilk et al., 2012; Fischer et al., 2013; van der Poel, 2012). Amniotic fluid (e.g., swab samples from the skin of malformed newborn animals) and placenta are also suitable as diagnostic material. Detection of SBV-RNA in the meconium is also possible although the detection rate in this material is lower (Wernike et al., 2014). Confirmation of infection could be made by viral isolation and detection of SBV sequences using RT-qPCR in serum and tissues.

Molecular diagnosis of SBV infection relies on the detection of the viral genome by RT-PCR. A SBV diagnostic assay was initially developed by the FLI (Bilk et al., 2012; Fischer et al., 2013). The technique is based on the simultaneous amplification of a SBV gene and an endogenous or exogenous added gene, which is used as an internal positive control (IPC) to ascertain RNA integrity and the absence of PCR inhibitors. Primers amplifying a part of the L gene segment were first used for the detection of the SBV genome (Hoffmann et al., 2012). A protocol targeting the S

segment was latter optimized and showed higher sensitivity (Bilk et al., 2012; Fischer et al., 2013). The latter protocol was implemented in most laboratories in Europe. Brain samples from aborted or stillborn lambs, kids and calves have mainly been used for the diagnosis of SBV. Studies have shown that samples from the cerebrum, external placental fluid and the umbilical and spinal cord are suitable for the detection of SBV (Bilk et al., 2012; van der Poel, 2012) and that the highest concentration of SBV RNA is found in the brainstem (De Regge et al., 2013). The detection of SBV proteins and RNA in paraffin sections can be performed by immunohistochemistry and in situ hybridization, respectively (Tauscher et al., 2017; Gerhauser et al., 2014).

To date several serological and molecular assays have been developed; among them, the N protein-based ELISAs and the S segment targeting RT-qPCR are widely applied (Bréard et al., 2013; Bilk et al., 2012). Both virus neutralization and an indirect immunofluorescence assays have been used for SBV antibody detection (Loeffen et al., 2012; Mansfield et al., 2012; Wernike et al., 2015). As serological evidence of this disease is emerging from areas outside of Europe, in some cases where other Simbu serogroup viruses are endemic, a need for additional diagnostic assays are needed. Therefore, in view of this scenario, the present study was conducted with three objectives: 1. Compare the performance of different SBV proteins (whole virus lysate, Gc, Gn, and N) as antigens in an indirect ELISA platform; 2. Evaluate the efficiency of three commercial nucleic acid (NA) extraction kits (Applied Biosystems, Cador/Qiagen, and GeneReach) in extracting SBV RNA from cell culture supernatant; 3. Compare the efficiency of gene-specific RT-qPCRs (S, M, L) in both single- and multiplex format.

4.3 Enzyme-linked immunosorbent assay (ELISA)

4.3.1 Materials and methods

4.3.1.1 Preparation of ELISA antigens

SBV glycoproteins Gn, Gc, and N protein were expressed in baculovirus with a C-terminal 6x histidine and purified in Ni-NTA Superflow resin (Novagen, Rockland, MA) as it was described in chapter 3. For the whole virus protein preparation, SBV was cultivated in Vero cells. Cells were grown for 36 h at 37°C and 5% CO_2, then infected with SBV at a multiplicity of infection (MOI) of 1. After 1 h incubation at 37°C and 5% CO_2, inoculum was aspirated and replaced by 1 mL of Dulbecco's minimum essential media (DMEM) with 10% FCS. Cells were incubated at 37°C and 5% CO_2 for 36 h, then the media was removed, cells were washed once with 1x PBS. Non-infected Vero cells, grown and handled under similar conditions, were used for the preparation of control antigen. Cells were freeze-thawed two times and the material was centrifuged at 1500 x g for 15 minutes. The pelleted cell debris was dissolved in 1/100 of the starting volume in PBS and stored in −80°C until used. The cell free viruses from the supernatants were concentrated using an Amicon filter (Fisher Scientific). To solubilize and inactivate virus, the concentrated virus and the cell bound virus fractions were treated by adding Triton X-100 (Merck) to a final concentration of 0.1% (v/v). The solutions were then sonicated in two cycles of 1 minute and incubated with agitation at room temperature for 15 minutes between the sonication steps, clarified by centrifugation for 15 minutes at 1500 × g and then stored in −80°C until used.

4.3.1.2 ELISA procedure

In order to measure the performance of each SBV antigen (baculovirus expressed SBV glycoprotein Gn, Gc, N protein, and whole virus lysate) in detecting anti-SBV antibodies in serum,

an indirect ELISA platform was used according to previously described protocols (Breard et al., 2013a; Faburay et al., 2014) with minor modifications. Briefly, two concentrations (100 and 200 ng/well) of each antigen and un-infected Sf9 and Vero cell lysates (negative controls) were used to coat plates and incubated overnight at 4 °C. The next day, wells were blocked with PBS (pH 7.4) containing 0.1% Tween and 1% skim milk for 15 min at 37°C. After washing three times with 0.1% Tween-20 in PBS, 100 µl of 1:200 diluted bovine sera from a previous study with VNT titers of 1:128 and <1:4 were used as positive and negative controls and incubated at 37°C for 1 h. All subsequent washing steps were carried out three times. After washing, plates were incubated with goat anti-bovine IgG–HRP conjugate for cattle (Thermo Scientific, IL), diluted 1:500 in blocking solution, at 37°C for 1 h. After another wash step, 100 µl of substrate buffer containing 0.1 mg/ml 3,3',5,5'-tetra-methylbenzidine (TMB) (Thermo Scientific, Rockford, IL) and hydrogen peroxide (H_2O_2) was added and the plates were incubated in the dark for 25 min. The reaction was stopped with 2 M H_2SO_4, and optical densities (OD) were measured at 450 nm using a microplate reader (Fluostar Omega, BMG Labtech, NC). The OD value of each sample and respective control sera was calculated as the difference between the OD values of the virus antigen well and the control antigen well (net OD values). The cutoff OD value was determined by addition of 2 standard deviations (SD) to the mean OD value of 0 dpi sera from the experimental animals.

4.3.1.3 Statistical analysis

The comparison of the reactivity of SBV lysate to the three BSV proteins (Gc, Gn, and N) was performed using the un-paired t-test with Welch's correction in GraphPad Prism version 8.0 (GraphPad Software).

4.3.2 Results

4.3.2.1 Performance of SBV antigens in an indirect ELISA

The suitability of whole SBV lysate and different recombinant SBV proteins (SBV-N, SBV-Gc, SBV-Gn) for use as antigen for detecting SBV-specific antibodies was evaluated using an indirect ELISA format. Two antigen concentrations (100 and 200 ng/well) and two SBV-positive cattle sera (C-60, C-66) from a previous study were used. SBV-N and SBV-Gc proteins were the most sensitive antigens (average net OD values for both sera: Gc = 1.05 (100 ng), 0.99 (200 ng); N = 0.97 (100 ng), 0.91 (200 ng); followed by the SBV-Gn antigen Gn = 0.81 (100 ng), 0.65 (200 ng). In contrast, the whole SBV lysate was not very immunogenic with net OD values of 0.36 (100 ng) and 0.40 (200 ng) (Fig. 4.1).

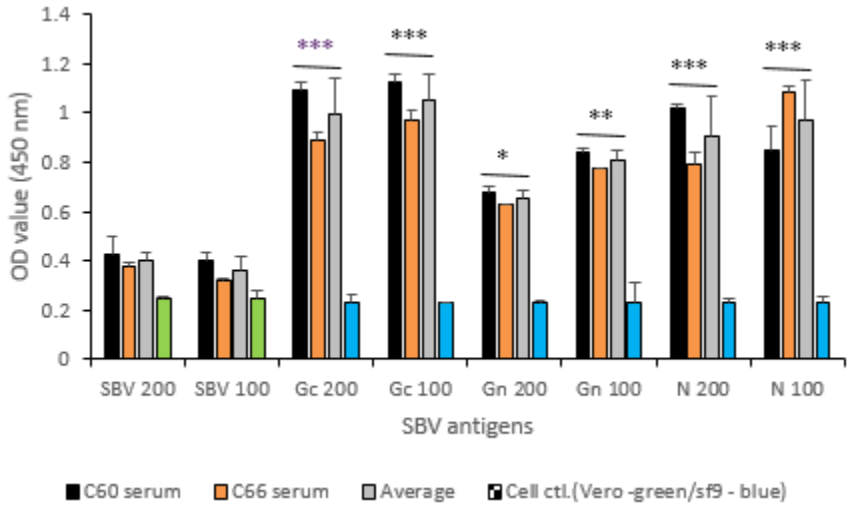

Figure 4.1 Net OD value of each antigen at two concentrations (100 ng and 200 ng) using SBV positive sera C60 and C66. SBV = whole virus lysate; Gc = glycoprotein C; Gn = glycoprotein N; N = nucleoprotein. Data represent the average values ± SEM of four proteins. Statistical analysis was performed using the un-paired t-test with Welch's correction. Significant differences between two proteins (SBV vs Gc; SBV vs Gn; SBV vs N) are indicated *p<0.05; **p<0.01 and ***p<0.001).

4.3.2.2 Analytical sensitivity and specificity of the Gc-specific ELIA

A total of 24 cattle sera, 12 SBV neutralizing antibody positive and 12 negative sera were used to assess the agreement between SBV Gc-specific ELISA and the virus neutralization assay. Ten out of 12 VNT positive sera were found to be positive by the Gc ELISA resulting in a sensitivity of 83.3% and all (12 out of 12) VNT negative sera were tested negative implying a 100% specificity. Therefore, the SBV Gc-specific ELISA has lower sensitivity than the VNT (Table 4.1).

Table 4.1 Comparison between SBV Gc-specific ELISA and VNT.

		Virus neutralization test (VNT)		
		Positive	Negative	total
ELISA	Positive	10	0	10
	Negative	2	12	14
	Total	12	12	24

4.3.2.3 Anti-SBV antibodies cross-reactivity to RVFV

The cross-reactivity of SBV-specific antibodies from sheep and cattle were checked against the Rift valley fever virus (RVFV) N antigen using a RVFV competitive ELISA kit (Upreti, et al. 2018). None of the SBV-specific sera were found to be cross-reactive to RVFV N antigen, indicating the lack of conserved antigenic epitope within the N proteins of Schmallenberg and RVF viruses (Fig. 4.2).

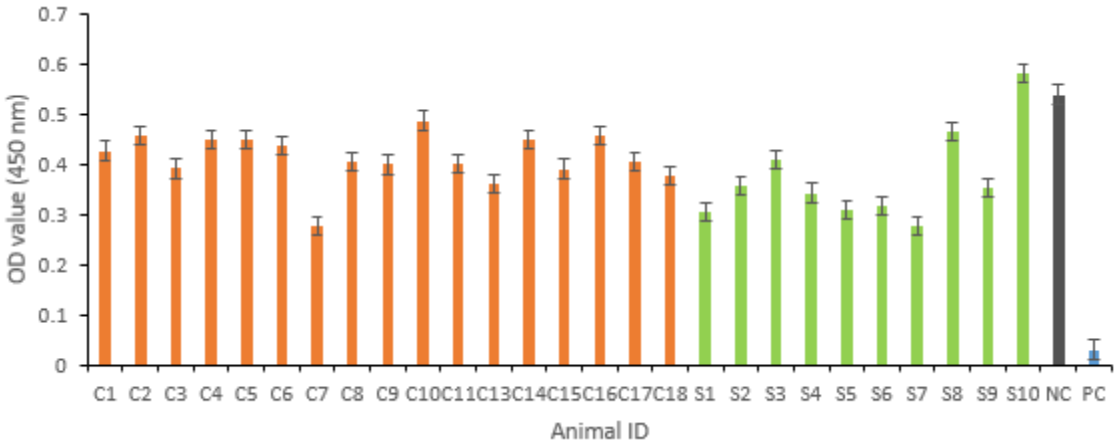

Figure 4.2 Summary of the RVFV competitive ELISA result using SBV-positive sera from cattle (C1-C18) and sheep (S1-S10). C = cattle; S = sheep; NC = negative control; PC = positive control.

4.4 Nucleic acid extraction method

4.4.1 Materials and methods

For the evaluation of different nucleic acid extraction protocols, the aqueous phase of trizol-inactivated, serial 10x dilutions of SBV-infected Vero cell culture supernatant was extracted using the following three magnetic beads extraction kits: 1. MagMax, Applied Biosystems using Beckman magnetic particles; 2. MagAttract 96 cador Pathogen kit, Qiagen; and 3. Taco DNA/RNA extraction kit, GeneReach. Extractions were performed on the BioSprint 96 automated magnetic particle processor (Qiagen) and detailed in Table 4.2. MagMax extraction was performed according to the manufacturer's instruction. Briefly, 100 µL of clarified lysate was transferred to the first row of a 96-well processing plate followed by addition of 90 µL of 100% isopropanol. The samples were mixed by pipetting before adding 10 µL of magnetic beads. Four subsequent washes were performed (150 µL each of wash-1, 2x, and wash-2, 2x) and the RNA was eluted in 30 µL of elution buffer at 65 °C. The MagAttract 96 cador extraction was performed as follows: 100 µL of the clarified lysate was transferred to a 96-well, deep-well plate containing 25 µL of

magnetic beads, 100 µL buffer VXL, 400 µL buffer ACB, and 100 µL PBS. Mixing of the reagents in the lysate plate was followed by 4 washes on the automatic processor. Wash 1 and 2 (700 µL/well of AW1 each), wash 3 (700 µL/well of AW2), and wash 4 (750 µL/well of 100% molecular grade ethanol) (Fisher Scientific, IL, USA). RNA was eluted with 60µL/well elution buffer. The GeneReach extraction protocol used 100 µL of lysate, 50 µL of magnetic bead, 300 µL of lysis buffer, and 200 µL of isopropanol and four wash steps. Wash 1 and 2 (450 µL/well of wash A), wash 3 (450 µL/well of wash B), and wash 4 (450 µL/well of 100% molecular grade ethanol). RNA was eluted with 60 µL/well elution buffer.

Table 4.2 Reagents used for nucleic acid extraction protocols.

	Quantity of reagent (µl)/well		
Reagents	Applied Biosystems	GeneReach	Cador
Lysis buffer/VXL	none	300	100
Beads	10	50	25
Isopropanol/ACB	90	200	400
Sample (aqueous)	100	200	200
Wash-1	150	450	700
Wash-2	150	450	700
Wash-3	150	450	700
Wash-4	none	450	750
Elution buffer	30	60	60

4.4.1.1 SBV RT-qPCR

SBV-RNAs obtained from the three extraction protocols were quantified by an S segment-specific, RT-qPCR as described previously (Bilk et al. 2012). The RT-qPCR assay was optimized with the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, CA, USA) using 5 µL RNA template in a total reaction volume of 25 µL and 42 cycles of amplification.

4.4.2 Results

SBV-RNA extraction was done from ten-fold serial dilutions (10^{-1} through 10^{-6}) of cell culture supernatant with $4x10^5$ pfu/mL. The extraction efficiency of each protocol was determined by the Ct values generated from the S-segment-specific RT-qPCR run and is indicated in table 4.3. Overall, the GeneReach protocol had the highest efficiency (lowest Ct) followed by the Cador/Qiagen and the Applied Biosystems protocols. At a high dilution, both the Cador/Qiagen or and GeneReach extraction kits showed a similar performance efficacy.

Table 4.3 Comparison of RNA extraction protocols using SBV serum (10-fold dilutions).

	1.00E-01	1.00E-02	1.00E-03	1.00E-04	1.00E-05	1.00E-06
AB	25.97 ± 0.3	33.49 ± 0.5	37.04 ± 0.8	37.89 ± 0.9	38.07 ± 0.9	ND
GR	21.21 ± 0.9	23.88 ± 0.5	28.44 ± 0.3	33.26 ± 0.4	38.75 ± 0.8	ND
Cad	26.31 ± 0.5	28.92 ± 0.8	30.57 ± 0.6	34.52 ± 0.7	38.73 ± 0.8	ND

AB = Applied Biosystems; GR = GeneReach; Cad = Cador/Qiagen; ND = not detected; Ct values listed

4.5 Reverse transcription real time PCR

4.5.1 Materials and methods

4.5.1.1 *In vitro* transcribed RNA

In vitro transcribed (ivt) SBV RNA (S, M, & L segments) and exogenous internal positive control (XIPC) RNA were generated for singleplex and multiplex RT-qPCR optimization. Specific sequences of each of the SBV RNA segments and XIPC [insert sizes of S (522), M (612), L (552) and XIPC (1068)] were chemically synthesized and cloned into Bluescript II SK plasmid vectors. Plasmids were linearized using HindIII restriction enzyme digestion and SBV-specific and XIPC RNAs were transcribed using MEGAscript T7 Transcription Kit (Thermofisher Scientific). In vitro transcription was performed according to the manufacturer's instructions. Amplicons were quantified using a spectrophotometer and sizes verified by gel electrophoresis. The stock concentration of each segment was: S ($7x10^{10}$ pfu/mL); M ($8.8x10^{11}$ pfu/mL); L ($6x10^{11}$ pfu/mL). Ten-fold dilutions of each ivt RNA was prepared, aliquoted and stored in -80 °C.

4.5.1.2 RT-qPCR

Ten-fold serial dilutions of *in vitro* transcribed SBV S, M, and L RNA were used for PCR standard curve development using *TaqMan* primers and probes for S, M, and L as described by Fischer et al (2013) (Table 4.4) in singleplex and multiplex formats. Probes used: S-segment with the reporter FAM; M-segment with reporter Cy5 and L-segment with reporter HEX at their 5' ends and IBFQ (Applied Biosystems) at their 3' ends. Amplification was carried out in a 20 µl volume reaction. Briefly, 10 µl 2x qScript XLT One-Step RT-qPCR Tough Mix master mix (Quanta Biosciences, Beverly, MA), 200 nM primer pairs for each of SBV segment and probes (0.4 µl

each). For one reaction, 5 µl RNA template was added. The reactions were incubated at 50° C for 20 min, followed by 95° C for 5 min, 45 cycles of 95°C for 10 sec, and 60°C for 1 min.

Table 4.4 SBV-specific oligonucleotides used in the study

Primers and probes	Nucleotide sequence	Position	Target segment
SBV-S-Forward	TCA GAT TGT CAT GCC CCT TGC	382-469	S
SBV-S-Reverse	TTC GGC CCC AGG TGC AAA TC		
SBV-S-FAM	FAM-TTA AGG GAT GCA CCT GGG CCG ATG GT-BHQ1		
SBV-M1-Forward	TCA ATT CAG CAA GTA ACA TAC AAT GG	1690-1827	M
SBV-M1-Reverse	CGT GGT CTG TCT TGG TTG ATG		
SBV-M1-Cy5	Cy5-AAG CAC TGG CCC GAA GTT TCA CCT-BHQ1		
SBV-L-1.2-Forward	TCA GAA TTG CCG TTT GAT TTT GAA G	361-468	L
SBV-L-1.4-Reverse	GTT GAG CGG CCC AAA TAT TTC C		
SBV-L1-HEX	HEX-TCA TCC GTG CTG ACC CTC TGC GAG-BHQ1		

4.5.1.3 Reverse transcriptase Real time PCR efficiency

Ideally, the amount of PCR product will perfectly double during each cycle of exponential amplification; that is, there will be a 2-fold increase in the number of copies with each cycle. This translates to a reaction efficiency of 2. An efficiency of 2 corresponds to a slope of 3.32 in a standard curve. Amplification efficiency is also frequently presented as a percentage, that is, the percent of template that was amplified in each cycle. An efficiency close to 100% is the best indicator of a robust, reproducible assay. Typically, desired amplification efficiencies range from 90% to 110% (Tichopad et al. 2003; Pfaffl et al. 2001).

4.5.2 Results

4.5.2.1 Singleplex/multiplex Reverse transcription Real time PCR

In-house *in vitro* transcribed SBV RNAs (for each segment: S, M, and L) were used to evaluate the efficiency of singleplex and multiplex real time RT-PCR. The PCR efficiency was determined by the CFX manager software 3.0 (Bio-Rad Laboratories Inc., Hercules, USA) for each SBV subgenomic RNA. The S gene had an efficiency of 131% (slope = 2.7) and 179% (slope = 2.3) in the singleplex and multiplex settings, respectively, which are outside of the recommended range. Also, the data points do not form a straight line. Amplification was not detected at the dilution 1.00E-8 and above. On the other hand, the M gene RT-PCR had an efficiency of 106% (slope = 3.2) and 96% (slope = 3.4) in the singleplex and multiplex settings, respectively (Table 4-4, 4-5). The data points form a straight line. The L gene RT-PCR had an efficiency of 103% (slope = 3.2) and 99% (slope = 3.3) in the singleplex and multiplex settings, respectively. The data points form a straight line similar to the M gene assay. Amplification was not efficient at dilution of 1.00E-3 and lower for both the M and L gene assays. In both singleplex and multiplex, M and L segment RT-PCR had higher efficiency than the S segment assay (Table 4.5, 4.6).

Table 4.5 Singleplex real time RT-PCR using 10-fold dilutions of a mixture of SBV ivt RNA templates (S, M, and L).

	1.00E-03	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10
SBV-S	22.0±0.37	25.69±1.5	29.53±1.5	32.84±1.6	36.31±1.6	ND	ND	ND
SBV-M	BN	23.4±0.08	26.7±0.05	29.4±0.16	33.3±0.44	36.2±0.87	ND	ND
SBV-L	BN	22.0±0.1	25.2±0.03	28.4±0.07	31.9±0.17	34.8±0.28	ND	ND

BN = background noise; ND = not detected

Table 4.6 Multiplex real time RT-PCR using 10-fold dilutions of a mixture of SBV ivt RNA templates (S, M, and L).

	1.00E-03	1.00E-04	1.00E-05	1.00E-06	1.00E-07	1.00E-08	1.00E-09	1.00E-10
SBV-S	24.5±1.1	28.0±0.24	30.9±0.33	33.6±0.47	35.3±0.26	ND	ND	ND
SBV-M	BN	22.1±0.07	25.3±0.03	28.7±0.28	31.7±0.63	36.0±1.3	ND	ND
SBV-L	BN	21.0±0.04	24.3±0.06	27.5±0.14	30.7±0.08	34.1±0.47	ND	ND

BN = background noise; ND = not detected

Multiplex RT-qPCR for SBV resulted in favorable amplification of the L and M genes with improved Ct values for M gene detection (1.4 Ct) and L gene (1.0 Ct) (Table 4.7) over the dynamic range of the PCR assays. A slight reduction in the amplification of the S gene in the multiplex setting was observed when compared to the singleplex assay (1.2 Ct.) (Table 4.7).

Table 4.7 Amplification difference between singleplex and multiplex SBV S, M, and L segment PCRs.

		IVT RNA dilution					
		1.00E-03	1.00E-04	1.00E-05	1.00E-06	1.00E-07	
Target				Ct value			Ave. Δ Ct
S	SP	22.09	25.69	29.53	32.84	36.31	
	MP	24.56	28.01	30.91	33.66	35.34	
	Δ Ct	**-2.47**	**-2.32**	**-1.38**	**-0.82**	**0.97**	**-1.20**
M	SP		23.40	26.78	29.99	33.31	
	MP		22.12	25.34	28.71	31.79	
	Δ Ct		**1.28**	**1.53**	**1.28**	**1.52**	**1.42**
L	SP		22.06	25.24	28.40	31.94	
	MP		21.08	24.31	27.55	30.7	
	Δ Ct		**0.98**	**0.93**	**0.85**	**1.24**	**1.00**

4.6 Next generation sequencing of SBV

4.6.1 Materials and methods

4.6.1.1 RT-qPCR assay for sequencing

Total RNA was extracted from 250 µL of serum or cell culture supernatant inactivated with 750 µL of Trizol (Invitrogen). Reverse transcription was initiated with the forward primers only but the PCR amplification was carried out using both forward and reverse primes SBV_L1_F/R, SBV_L2_F/R, SBV_L3_F/R, SBV_M1_F/R, SBV_M2_F/R and SBV_S1_F/R, respectively (Table 4.8). Eight microliter RNA was mixed with 1 µL primer (2 mM) and denatured at 65 °C for 5 min. Reverse transcription was carried out with the SuperScript III First-Strand Synthesis System for RT-PCR (Thermofisher Scientific) following the manufacturer's protocol. cDNA was amplified with the Platinum™ PCR SuperMix High Fidelity (Thermofisher Scientific) with gene-specific primers (Table 4.8), following the manufacturer's protocol, using 7 µL cDNA in a 50 µL reaction volume. PCR was run on an Eppendorf Master Cycler using the following cycling profile: 1 cycle 94 °C/3 min., then 45 cycles [94 °C/30 s, 50-57 °C/30 s, 68 °C/4 min], followed by 1 cycle of 68 °C/10 min.

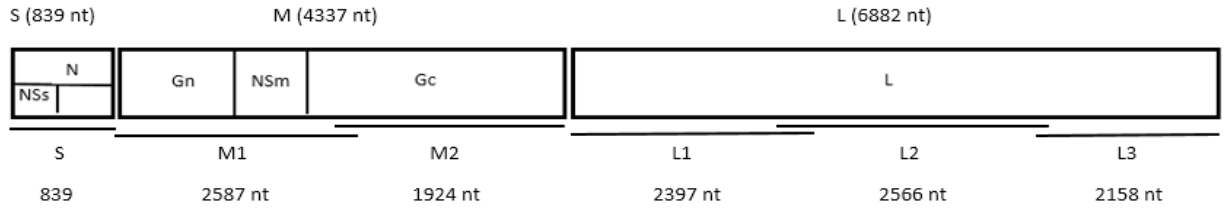

Figure 4.3 Schematic representation of RT-PCR amplification/sequencing strategy to obtain full-length SBV genome. The size of each segment is indicated and the respective genes are represented. Black lines located below each segment represent the overlapping amplicons used for the amplification sequencing of the SBV genomes.

Table 4.8 **Primers used for cDNA synthesis and PCR of the viral RNA segments L, M, and S.** Primers were designed based on the genome of SBV isolate B80/11-4 (GenBank accession numbers HE649912, HE649913, HE649914 for L, M, and S segment, respectively).

SBV RNA segment	Orientation	Primer name	Primer sequence
L1	Forward	SBV_L1_F	AGTAGTGTACCCCTAATTACAA
	Reverse	SBV_L1_R	TGCAGTTTGTTTTCTAGGTGTTG
L2	Forward	SBV_L2_F	TGGTTTGAAGGCTATGTATCACT
	Reverse	SBV_L2_R	CCCAATCGTTGCATCTCCAC
L3	Forward	SBV_L3_F	AGCTTCTGTATAATGAACGACCC
	Reverse	SBV_L3_R	GTAGTGTGCCCCTAATTACATGA
M1	Forward	SBV_M1_F	CCACAATCAAAATGCTTCTCAACATTG
	Reverse	SBV_M1_R	CGAATGGGCTTTAGCTGAAACATAG
M2	Forward	SBV_M2_F	ACCATCATGCAACGTGAAGAG
	Reverse	SBV_M2_R	AGTAGTGTTCTACCACATGAAAAACTTCT
S	Forward	SBV_S_F	CACAACGGAATGCAGCTACA
	Reverse	SBV_S_R	GAAGCCTTGCAGTATAATGGTG

After RT-PCR, the nucleotide sequences of the L, M, and S segment, respectively, were determined in both directions by direct sequencing of cleaned PCR products obtained from the three SBV collections (FLI/KSU serum and cell passaged SBV).

4.6.1.2 Next generation sequencing and analysis

Using equimolar volume of each DNA segment, the sequencing library was prepared from 1 ng of DNA for each of the isolates using the Nextera XT-DNA library preparation kit (Illumina, CA). Sequencing was performed using standard protocols on the Illumina MiSeq instrument, with 150-bp paired-end reads.

FASTQ files derived from sequencing were imported into CLC Genomics Workbench version 7 (Qiagen) for analysis. De novo assembly was performed at 50% length similarly and 99% overall similarity to obtain contigs representing the L, M, and S segments of each sample. The only exception was the cell-grown SBV isolate sample, which failed to produce a contig corresponding to the S segment. For this individual sample, total reads were mapped to the BH80/11-4 S segment reference sequence at 50% length similarity and 75% overall similarity to determine the sequence of cell-grown SBV isolate S segment. Multiple sequence alignments were performed using ClustalW in MacVector.

4.6.2 Results

4.6.2.1 SBV sequence comparison

In order to facilitate full genome sequencing of SBV isolates for diagnostic purposes, we established a full genome sequencing approach for three different SBV isolates: FLI-serum, KSU-serum, and KSU-serum cell passaged. This approach was also undertaken to determine the molecular evolution of SBV when passaged in cattle and after one passage in cell culture. The "FLI-serum" sample was the inoculum used to infect cattle at KSU; the "KSU-serum" sample was the FLI-serum virus passaged once in cattle; and the "cell-passaged SBV" isolate sample was the KSU-serum isolate passaged once in cells. The genome of the first SBV strain BH80/11-4 detected in German cattle was used as a reference strain in the analysis.

The whole genome sequence of each sample was determined using Next Generation Sequencing (NGS). Equimolar concentrations of each PCR fragment from the L, M, and S segments of each sample were combined and subjected to NGS-specific library preparation and sequencing on a MiSeq NGS sequencer. The FLI-serum sample resulted in 6,560,214 paired-end reads, the KSU-serum isolate resulted in 2,374,340 paired-end reads, and the cell-passaged SBV isolate resulted

in 2,683,062 paired-end reads. FASTQ files containing these reads were then subjected to *de novo* assembly to determine the sequences of the L, M, and S segments of each sample. The only exception was the S segment of the cell-passaged SBV isolate, which required mapping reads to the BH80/11-4 reference sequence. For the FLI-serum isolate, the L, M, and S segments had an average read coverage of 63,632x, 71,075x, and 59,048x, respectively. For the KSU-serum isolate, the L, M, and S segments had an average coverage of 29,019x, 24,063x, and 20,724x, respectively. For the cell-passaged KSU isolate, the L and M segments had an average coverage of 30,677x and 33,928x, respectively, and the S segment had a maximum coverage of 21,307x (average coverage was not determined due to the difference in sequence determination strategy).

Nucleotide and amino acid differences were then determined for each of the SBV samples to determine molecular evolution of the virus upon different passage history (Table 4.9). The L segment contained a silent G to A mutation (nt 1827) in the RdRp coding sequence (CDS) found in the KSU-serum and cell-passaged KSU samples. The L segment also contained two additional silent mutations (T to C, nt 2154; T to A, nt 5736) in the RdRp coding sequence of all three samples when compared to the BH80/11-4 reference sequence. The M segment contained the most mutations when compared to the reference sequence. There were two silent mutations found in the Gn coding sequence of all three samples that differed from the BH80/11-4 reference sequence (T to C, nt 482; G to A, nt 836). All three samples also contained a mutation in the NSm coding sequence (G to A, nt 1117) that differed from the BH80/11-4 reference sequence and results in a serine to asparagine mutation. The Gc coding region contained four mutations, two (A to G, nt 2247; A to G, nt 4029) of which result in an amino acid change from a lysine to glutamate. Another silent mutation (T to C, nt 4230) was found in all three samples when compared to the BH80/11-4 reference sequence. A silent mutation (T to C, nt 3968) was found in the KSU-serum and cell-

passaged KSU serum isolates, but not in the FLI-serum sample. Only one mutation was observed in the S Segment and present only in the cell-passaged KSU serum sample (T to C, nt 51), which causes a valine to alanine substitution in the nucleoprotein coding region. However, it must be noted that this may be an artifact due to the fact that this nucleotide is located at the end of the S segment and the result may be influenced by the lower read coverage at this region. Additional studies will be necessary to determine if this is a true mutation found in this sample. Overall, these results show that the SBV genomes between each of the tested SBV samples have over 99.9% homology, indicating a low level of molecular evolution at this level of investigation.

Table 4.9 Comparison of consensus nucleotide sequences of SBV isolates (FLI-serum, KSU-serum, cell passaged KSU-serum) with respect to the reference strain BH80/11-4 (numbering based on BH80/11-4 reference sequence).

SBV RNA segment	Nucleotide position	BH80/11-4 Reference (GenBank)	SBV-FLI	SBV-KSU	Cell passaged SBV-KSU	Coding region	Amino acid change
L	1827	G	G	A	A	RdRP	Silent
L	2154	T	C	C	C	RdRP	Silent
L	5736	T	A	A	A	RdRP	Silent
M	482	T	C	C	C	Gn	Silent
M	836	G	A	A	A	Gn	Silent
M	1117	G	A	A	A	NSm	S > N
M	2247	A	G	G	G	Gc	K > E
M	3968	T	T	C	C	Gc	Silent
M	4029	A	G	G	G	Gc	K > E
M	4230	T	C	C	C	Gc	Silent
S	51	T	T	T	C	N	V > A

FLI – Friedrich Loeffler Institut; KSU – Kansas State University

4.7 Discussion

Laboratory testing for SBV is necessary to confirm clinical cases of SBV infection, especially malformation in fetuses. Blood samples collected from newborns before colostrum intake is a good sample to check antibodies and confirm fetal SBV infection. While both virus isolation and RT-PCR are the most suitable tests to confirm clinical cases, the former technique is time-consuming and the latter is more expensive than serological tests. At present, there is a commercial SBV antibody detection ELISA kit based on *E.coli* expressed recombinant N protein (Bréard et al., 2013b). Other commercial kits are also available from IDEXX laboratories and Boehringer Ingelheim animal health. An in-house ELISAs against Gc and Gc/Gn have been developed using recombinant Gc and Gc/Gn proteins (Wernike et al., 2017). The SBV N protein was also expressed in yeast and N-specific monoclonal antibodies were developed and tested for their immunoreactivity using different assays (Lazutka et al., 2014).

Since the nucleocapsid N protein is highly conserved among Simbu serogroup viruses, N-based ELISAs could also detect antibodies against other viruses in the serogroup, especially in areas where these viruses are endemic (Mathew et al., 2015; Blomstrom et al., 2014). Therefore, in our study we tried to evaluate the performance of indirect ELISAs based on SBV N, Gc and Gn proteins and a whole virus lysate for detection of SBV-specific IgG antibodies. As it has been reported before (Wernike et al., 2015), we found that both SBV-N and SBV-Gc are the most immunogenic antigens, followed by the SBV-Gn. On the other hand, the whole SBV lysate did not perform well in the indirect ELISA format. This is in contrast to a previous report (Naslund et al., 2014), where the whole virus ELISA demonstrated higher sensitivity than the N-based ELISA. This difference might be due to the different methods used to prepare the whole virus antigen.

The SBV-Gc ELISA had comparable specificity but lower sensitivity compared to the SBV-VNT. This agrees with previous reports (van der Poel et al., 2014). The decrease in the sensitivity of the ELISA may be due to the fact that it mainly detects IgG antibodies and not both IgM and IgG antibodies as in the VNT assay (Poskin et al., 2015).

The viremia phase for SBV is short-lived and the viral genome is only detectable for 2–6 days in the blood of infected cattle or sheep (Hoffmann et al., 2012; Wernike et al., 2012; Wernike et al., 2013). However, SBV-RNA may be detected for an extended period in the lymphoreticular system, particularly in the mesenteric lymph nodes (Wernike et al., 2012; Wernike et al., 2013). Molecular detection by RT-qPCR is an ideal method for detection of SBV. Several RT-qPCR assays were developed for the detection of different RNA segments of SBV: small (S), medium (M), and large (L). However, the RT-qPCR assay targeting the S and M segments developed by FLI has been extensively used in Europe for active surveillance and diagnostics. The viral load in different tissues after natural infection is very low, so an efficient nucleic acid extraction protocol is critical. Previously different nucleic acid extraction methods have been evaluated for the extraction of SBV RNA from semen samples and the highest analytical sensitivity was found for the Trizol LS reagent lysis protocol (Hoffmann et al., 2013). This had been further confirmed during a laboratory proficiency trial conducted in 28 veterinary diagnostic laboratories in Europe (Wernike et al., 2017). In the current study, we found that the GeneReach NA extraction kit yielded approximately 10 times more SBV RNA as compared to the Cador/Qiagen and Applied Biosystems extraction kits. Variation in the detection of the SBV RNA from bovine semen was reported in an inter-laboratory comparison and some laboratories reported false negatives; this was partially attributed to a suboptimal nucleic acid extraction methods (Wernike et al., 2016). Taking all the results obtained with 3 different NA extraction kits into account, the extraction conditions

for SBV RNA need to be established before doing SBV diagnostics. RT-PCR diagnostic assay in a singleplex and multiplex format were analyzed. Singleplex RT-PCR assay resulted in a better performance for the S gene detection (1.2 Ct) compared to multiplex assay. On the other hand, the multiplex assay favored amplification of the L and M segments with improved efficiency for both M and L segments detection by 1.4 and 1.0 Ct, respectively (Table 4.7). It was noted that the S gene RT-PCR assay is less specific at high dilutions.

Next generation sequencing (NGS) can be used to perform detailed comparisons between virus isolates and to understand the molecular evolution of a virus. In this study, we determined the full genome sequence of three SBV samples using NGS: FLI-serum, KSU-serum, and cell-passaged KSU-serum. The sequence data obtained were compared to the SBV reference sequence BH80/11-4 derived from viremic cattle serum in 2012. The S segment was highly conserved in all three isolates (FLI-serum, KSU-serum, cell-passaged KSU serum), with only one mutation found in the cell-passaged KSU serum sample that may be an artifact due to low coverage. This finding is in line with previous reports of a high genetic stability of the S segment in different SBV samples obtained from mammalian hosts and arthropod vectors (Kesik-Maliszewska et al., 2018). On the other hand, the L and M segments had a low rate of mutations among the three virus samples: three mutations in the L segment and 7 mutations in the M segment when compared to BH80/11-4. However, all three mutations in the L segment were silent, demonstrating the molecular stability of the RNA-dependent RNA polymerase at the level of this investigation. Of the seven mutations observed in the M segment, three resulted in amino acid changes (NSm nt 1117, S to N; Gc nt 2247, K to E; Gc nt 4029, K to E), indicating a higher overall mutational rate in this genomic region. Previously, it has been reported that after 32 passages in cell culture, SBV accumulated mutations in all 3 viral segments, with most of the mutations/substitutions located in the Gc protein

coding region of the M segment (Varela et al., 2013). Our results are consistent with these findings. Overall, our NGS results indicate a low overall mutational rate in SBV. Higher levels of SBV molecular evolution may be possible over more cell passages or among an entire livestock herd. Additional studies will be necessary to fully elucidate the specific pressures that drive the molecular evolution of SBV.

4.8 References

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